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In continuation of previous grants, a standard operating procedure is being developed for two methods for retrospective detection of exposure to sulfur mustard: an immunoslotblot assay and/or ELISA for DNA adducts in blood and skin and the modified Edman procedure for determination of adducts to the N-terminal valine in hemoglobin. In the first year of the new grant period, both procedures could already substantially be shortened, while their sensitivities were improved. Furthermore, exploratory research is performed aiming at the development of a fieldable immunochemical assay for sulfur mustard adducts with three proteins, i.e., hemoglobin, albumin, and keratin. A reliable synthetic route was developed for [^{14}C]sulfur mustard used in various experiments. In vitro exposure of human blood to 1 μM sulfur mustard was determined by LC-MS-MS analysis of the tryptic T5 peptide from albumin alkylated at cysteine-34. Treatment with base released 80% of the bound radioactivity as [^{14}C]thiodiglycol from keratin isolated from [^{14}C]sulfur mustard exposed human callus, which suggests that most of the adducts formed are esters of glutamic and aspartic acid residues. This opens the way for mass spectrometric detection of sulfur mustard exposure of skin by GC-MS analysis of thiodiglycol after hydrolysis of the esters and derivatization. Three partial sequences of hemoglobin, the T5 fragment from albumin, and three partial sequences of keratin were synthesized as haptens for raising antibodies, which contain adducted histidine, cysteine, and glutamine or asparagine, respectively. Several clones have been obtained using the three peptides containing adducted histidine as haptens, in addition to the clone 3H6 raised against a partial hemoglobin sequence containing adducted cysteine. Antibodies of these clones show specificity not only for sulfur mustard treated hemoglobin but also for treated keratin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

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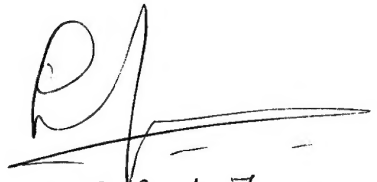

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SUMMARY

Within the framework of previous grants we have developed methods for retrospective detection of exposure to sulfur mustard which are based on the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays. In addition, LC-MS-MS and GC-MS analyses of the adducts were developed for validation of the immunochemical assays. Two methods had been sufficiently worked out to justify the development of a standard operating procedure (SOP) for application in a well-equipped field hospital, i.e., an immunoslotblot assay or ELSA of sulfur mustard adducts to DNA in human blood and skin, and a GC-NCI/MS determination of sulfur mustard adducts to the N-terminal valine in hemoglobin of human blood by using the modified Edman procedure. Development of these SOPs is one of the two major topics of the present grant. It is intended to develop two modes of immunoassay SOPs, one in which experimental time is as short as possible and another one in which sensitivity is the most important factor.

The modifications applied so far to the immunoslotblot assay were meant to simplify and to speed up the procedure while maintaining maximum sensitivity. In a later stage, modifications will be introduced in the ELISA test to speed up the procedure while accepting some decrease in sensitivity and accuracy. The procedure for DNA isolation from human blood for the immunochemical determination of N7-2-hydroxyethylthioethyl-guanine (N7-HETE-Gua) in DNA has been shortened substantially and can be carried out in ca. 4 h with only 300 µl of blood. In addition, the sensitivity of the immunoslotblot assay could be improved by UV crosslinking of the DNA in 1-µg quantities to the nitrocellulose filter and by direct measurement of the chemiluminescence with a luminometer. The lower detection limit was in the range of 3-13 N7-HETE-Gua/10^9 nucleotides. By using this modified procedure, treatment of double stranded calf thymus DNA to ≥ 2.5 nM sulfur mustard could be detected.

The modified Edman procedure for determination of sulfur mustard adducts to the N-terminal valine in hemoglobin including GC-NCI/MS analysis could be shortened to one working day without loosing sensitivity, by performing the Edman degradation reaction for 2 h at 60 °C instead of overnight at room temperature followed by 2 h at 45 °C. A substantial purification of the crude thiohydantoin was achieved by introducing a solid phase extraction step into the modified Edman procedure, which may allow us to process larger amounts of globin and consequently to detect lower exposure levels. Application of the thermodesorption/cold trap (TCT) injection technique in the GC-NCI/MS analysis of the final sample obtained after the modified Edman procedure led to a 3-fold decrease of the detection limit (from 0.1 to 0.03 µM) for in vitro exposure of human blood.

The retrospectivity of the diagnosis on the basis of protein adducts is superior to that on the basis of DNA adducts due to the generally much longer half lives of protein adducts. Therefore, exploratory research is performed aiming at the development of a fieldable sensitive immunochemical assay for sulfur mustard adducts with three proteins, i.e., hemoglobin, albumin, and keratin. In various series of these experiments, [^{14}C]sulfur mustard has advantageously been used. A synthetic route was developed for this compound which leads to more reproducible results than synthesis of the ^{35}S-labeled agent used in previous studies.

A properly protected building block of N1/N3-HETE-histidine was synthesized. This synthon was used in the solid phase synthesis of three partial sequences of hemoglobin containing an adducted histidine identified as a major alkylation site in the protein. Several clones have been obtained using these three peptides as haptens, in addition to the clone 3H6 which was raised against N-acetyl-S-HETE-cys$_{93}$ through leu$_{106}$-lys of the β-chain of hemoglobin. Antibodies of

these clones show specificity not only for hemoglobin alkylated with 50 μM sulfur mustard but also for alkylated keratin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Upon exposure of human blood to various concentrations of [^{14}C]sulfur mustard we found that a proportional amount (ca. 20%) was covalently bound to albumin. The major fragment formed by tryptic digestion was identified by LC-MS-MS analysis as the T5 peptide alkylated at cysteine-34, i.e., HETE-(A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C-P-F-E-D-H-V-K). This peptide was synthesized on a solid support as a hapten for raising antibodies against sulfur mustard treated albumin. LC-MS-MS analysis under multiple reaction monitoring conditions performed directly in a tryptic digest of albumin that was isolated from sulfur mustard treated human blood allowed to detect exposure to 1 μM of the agent.

Upon exposure of human callus (suspension in 0.9% NaCl; 20 mg/ml) to various concentrations of [^{14}C]sulfur mustard we found 15-20% of the added radioactivity covalently bound to keratin. Unfortunately, enzymatic digestion in order to identify specific alkylated sites did not give satisfactory results. Upon incubation with base, 80% of the bound radioactivity was split off as [^{14}C]thiodiglycol, which suggests that most of the adducts formed with keratin in human callus are esters of thiodiglycol with glutamic and aspartic acid residues. This result opens the way for sensitive mass spectrometric detection of sulfur mustard exposure of skin by GC-MS analysis of thiodiglycol, after hydrolysis of the esters and derivatization with, e.g., pentafluorobenzoyl chloride.

Two partial sequences of keratin K14 and one partial sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, by using a properly protected building block of glutamine or asparagine adducted with a 2-hydroxyethylthioethyl group at the amide function.

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I INTRODUCTION

The confirmed use of sulfur mustard, sarin and tabun in the Iran-Iraq conflict (1), the threat of chemical warfare in the Gulf War and the recent attacks with sarin by terrorists in Japan (2) have stressed the need of reliable methods to detect nature and extent of poisoning with chemical warfare agents. This need will increase in view of the expected further proliferation of chemical weapons in Third World countries. Moreover, these methods are also useful for the verification of alleged non-adherence to the Chemical Weapons Convention (3).

Within the framework of the previous grants (DAMD17-88-Z-8022 and DAMD17-92-V-2005) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (4-12). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays. LC-MS-MS analysis of hydrolysis products of alkylated proteins provides information on the identity and location of alkylated amino acids and therefore on the haptens that are needed to raise monoclonal antibodies against the adducts. The immunochemical assays can be performed on small samples, are highly sensitive, and can be applied "on site" when properly developed. Moreover, LC-MS-MS and GC-MS analyses are used to validate the immunochemical assays. In this way, it can be firmly established whether casualties have indeed been exposed to sulfur mustard, whereas dosimetry of the exposure will be a starting point for proper treatment of the intoxication. Moreover, the use of chemical warfare agents in the Iran-Iraq war has learned that reliable methods for verification of exposure to chemical warfare agents in alleged casualties and for identification of the agent are not available. Furthermore, experience with the casualties in the Iran-Iraq War and in other incidents (13) learned that biopsies or autopsies of alleged victims often become available several days or even weeks after alleged exposure. Very recently, the need for retrospective detection of exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome" (14). Our approach will provide the appropriate methodologies, since the adducts of sulfur mustard with DNA are stable *in vivo* for days and adducts with proteins are expected to last even for several months. The application of the two independent methods of analysis will provide evidence for alleged use of chemical agents with almost complete certainty.

In addition to the above-mentioned purposes, our assays can be used in a variety of other applications, e.g.:

- biomonitoring of workers in destruction facilities of the agent, or in laboratories involved with research on sulfur mustard,
- in animal experiments and in in vitro experiments with skin samples, e.g., in protective clothing penetration, skin decontamination and in inhalation studies, to establish an unambiguous relationship between external and internal dose (Langenberg *et al.*, 1994),
- in immunochemical staining techniques, in order to determine the location and frequency of adducts in inter- and intracellular structures of cells exposed to the agent,
- in forensic analyses in case of suspected terrorist activities.

The major results obtained in our previous studies are summarized as follows.

- Immunochemical methods developed in this study are superior for detection of DNA adducts in human white blood cells and skin; immunoslotblot assays enabled detection of an exposure of human blood to 70 nM sulfur mustard and exposure of human skin to air saturated with sulfur mustard vapor at 27 °C for only 1 s ($Ct \approx 18$ mg.min.m^{-3}).
- These methods are complemented in a valuable way by a procedure for LC-MS-MS analysis of the corresponding guanine adduct in urine (10).

- Immunochemical detection of protein adducts has led to encouraging results, achieving adduct detection after exposure of human hemoglobin to 50 μM sulfur mustard, but needs further development.
- Very promising results have been obtained for GC-NCI/MS and LC-MS-MS analyses of protein adducts (9,11); particularly, a modified Edman procedure for selective cleavage of the alkylated N-terminal valine in hemoglobin with pentafluorophenyl isothiocyanate detects exposure of human blood to sulfur mustard concentrations as low as 0.1 μM by means of GC-NCI/MS after further derivatization (9).
- Both the immunochemical and the mass spectrometric approach have been shown to be viable, based on positive analyses of blood samples taken in 1988 from Iranian casualties more than three weeks after exposure (12).
- The feasibility of LC-MS-MS analyses in tryptic digests of globin isolated from sulfur-mustard-treated human blood has been shown for identification of 5 alkylation sites within the tertiary structure of the protein (8).

In continuation of this work, studies along two lines are being performed in the present agreement. Firstly, two methods were sufficiently developed as a result of our efforts within the context of the former agreement, in order to justify the development of two standard operating procedures to be applied in a well-equipped field hospital, i.e.,

- the immunoslotblot assays of sulfur mustard adducts to DNA in human blood and skin and
- the GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin of human blood.

Secondly, immunochemical detection of protein adducts still needs further development, but is highly interesting since analysis of these longer lasting adducts enables retrospective detection of exposure during a longer period of time and, consequently, allows also to detect long-term low-level exposure to the agent. Further exploratory research is performed in the present agreement aiming at the development of a fieldable and sensitive immunochemical assay for sulfur mustard-protein adducts.

(i) The development of standard operating procedures for use under field conditions of the two methods includes optimization of the methods and validation of the procedures.

The immunochemical assay involves a number of steps which should be simplified and minimized as much as possible for application under field conditions. The isolation of DNA is rather laborious and technically complicated. Modifications to simplify and speed up this procedure have been introduced. In addition, improvements have been achieved in both the immunoslotblot procedure and the signal detection resulting in a higher sensitivity. Up to now, adducts have been detected in human blood and in skin biopsies. Since the skin is the critical target for most exposures to sulfur mustard, one might argue that the procedure should primarily be based on detection of adducts in skin biopsies. However, since taking skin biopsies is more invasive than taking blood samples, immunochemical field procedures for both targets are developed.

Modifications have also been introduced into the modified Edman procedure and the subsequent GC-NCI/MS analysis making the mass spectrometric method more rapid, simple and sensitive.

After some additional attempts to optimize the two methods, standard procedures will be set up which will be validated in animal experiments to be carried out in the 2nd and 3rd year of the agreement. The two procedures will be used in the same sets of blood samples at increasing time intervals after intravenous administration of sulfur mustard at various doses in order to demonstrate that the procedures are mutually confirming, to demonstrate that the results

depend on the dose, and to obtain data on the persistence of the various adducts. Hairless guinea pigs as well as marmosets[1] are proposed as experimental animals, allowing interspecies comparison. Finally, the practical applicability of an assays will be demonstrated by performing the developed procedures at another institute, i.e., the US Army Medical Research Institute of Chemical Defense.

(ii) The further exploratory research on detection of protein adducts is based on the promising results obtained with hemoglobin adducts in the previous agreement and on the systematic approach for immunochemical analysis of adducts which has evolved from these results. This approach involves the following steps:

- semi-quantitative analysis of amino acid-adducts after acidic and protease-catalyzed hydrolysis,
- exploratory LC-MS-MS analysis of adducts in peptides obtained by tryptic hydrolysis of the exposed protein, which will allow the determination of the site of alkylation in the adducted peptides,
- molecular modeling of the adducted protein based on the above-mentioned analyses, which will give a lead to the synthesis of the most appropriate haptens, i.e., similar to sequences at the outer surface of the protein,
- synthesis of synthons derived from adducted amino acids which are suitable for solid phase synthesis of peptide haptens,
- synthesis of multiple haptens for use in immunization experiments,
- development of immunochemical assays based on the simultaneous use of several monoclonal antibodies which will cover various adducts in an adducted protein.

The first two items will also provide guidance as to which amino acids should be used for quantitative GC-MS or LC-MS-MS analysis, in order to verify immunochemical assays.

The development of immunochemical analysis of protein adducts is aimed at three types of proteins, i.e., hemoglobin, albumin, and keratins in the skin. In this order, the accessibility of the adducts for immunochemical analysis is supposed to increase. Whereas hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma. Keratins in the skin, especially those in the stratum corneum, are directly accessible for reagents, which should give prospects to detect adducts by way of reagents sprayed on the skin.

Although rather strict structural demands have to be made upon the haptens to which antibodies against protein adducts are raised, cross reactivity among different adducted proteins cannot be excluded *a priori*. Therefore, the antibodies obtained against one adducted protein will be tested for cross reactivity with the two other proteins exposed to sulfur mustard.

For detection of hemoglobin adducts, it has been attempted to further improve the sensitivity of the immunochemical assay by using the monoclonal antibodies obtained in the previous agreement which were raised against S-HETE-cys_{93} of the β-chain of human hemoglobin. In addition, a synthon derived from adducted N1/N3-histidine was synthesized which was found to be the most abundant adduct formed in hemoglobin after exposure of human blood to sulfur mustard (7). Partial sequences of hemoglobin containing an adducted histidine identified as an alkylation site for sulfur mustard (7,8) were synthesized by using this building block. These peptides were applied for raising antibodies. Studies on the development of an immunochemical assay based on these antibodies will be performed in the second and third year of the agreement.

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[1] If it is not possible to obtain permission from the "TNO Committee for Animal Care and Use" to perform studies with a limited number of primates, the experiments will be carried out with rats.

Studies have also been performed in order to develop an immunochemical assay for the detection of sulfur mustard adducts with albumin, which is the most abundant protein in plasma. Alkylation of the protein was studied by using ^{14}C-labeled agent. One of the alkylated peptides formed upon treatment with trypsin of albumin which was exposed to sulfur mustard, i.e., the fragment T5 containing an alkylated cysteine, could sensitively be detected in the tryptic digest with LC-MS-MS analysis. The feasibility of this approach will be investigated for retrospective detection of exposure to sulfur mustard during next year. During this period a Q-TOF-mass spectrometer will become available in TNO Prins Maurits Laboratory, by which much more structural information of peptide analytes can be acquired than by LC-MS-MS analysis while maintaining the same sensitivity. Furthermore, this alkylated peptide has been synthesized and will serve as a hapten for raising antibodies.

To the best of our knowledge, no attention has been paid so far to the analysis of adducts of alkylating reagents with proteins present in the skin. However, the skin is a major target for chemical warfare agents, such as the vesicant sulfur mustard. The primary site of exposure in the skin will be keratin, which is the most abundant protein present in the human epidermis and stratum corneum. In order to explore the feasibility of detection of keratin-sulfur mustard adducts, human callus was exposed to ^{14}C-labeled agent. The major part of the radioactivity was readily removed from the protein upon treatment of the isolated keratin with alkali, suggesting that most of the adducts formed in keratin are esters of glutamic acid and aspartic acid. A procedure for a sensitive mass spectrometric analysis of thiodiglycol formed from the amino acid esters upon alkali treatment will be worked out in the next year of the agreement.

We expect that alkylation proceeds mainly in the terminal regions of keratins. Three peptides were synthesized which consist of partial sequences of end domains in the two most abundant keratins present in human skin and contain an adducted glutamine or asparagine. Immunochemical studies in which these peptides are used as haptens will be performed in the second and third year of the agreement.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously: Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester (3), 2-(2-aminoethylthio)ethanol (7), and sulfur mustard-d_8 (7). The monoclonal antibody 2F8, directed against N7-HETE-guanine (N7-HETE-Gua) in DNA was the same as described previously (7).

Thionylchloride was purchased from Janssen Chimica (Tilburg, The Netherlands) and distilled before use. N-methylmorpholin (NMM, Janssen Chimica) was distilled from NaOH at atmospheric pressure before use. N-methylpyrrolidone (NMP, Aldrich Chemie, Bornem, Belgium) was vacuum distilled under a nitrogen atmosphere before use.

9-Fluorenylmethoxycarbonyl (Fmoc) amino acids purchased from Novabiochem (Läufelfingen, Switzerland) were of the L configuration, bearing the following side chain protecting groups: *tert*-butyl (tBu) for aspartic acid, glutamic acid, serine and threonine, trityl for histidine and asparagine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl for arginine, and *tert*-butyloxycarbonyl (Boc) for lysine. Tentagel S AC (Rapp Polymere, Tübingen, Germany) was used as a resin (40-60 mg per peptide, 10 μmol of Fmoc amino acid loading).

The following commercially available products were used:
diethanolamine, gelatin, glycine, poly(ethylene glycol) (PEG 4000), poly(ethylene glycol) (PEG 20,000), proteinase K (Merck, Darmstadt, Germany); acetonitrile (Baker Chemicals, Deventer, The Netherlands); human serum albumin (HSA), pentafluorophenyl isothiocyanate (PFPITC), borane tetrahydrofuran complex solution (1.0 M), sodium ethoxide solution, pentafluorobenzoyl chloride (Fluka, Buchs, Switzerland); N-Boc-1-*tert*-butyl-L-glutamate (Boc-Glu-OtBu), dl-dithiothreitol (DTT), iodoacetic acid sodium salt, TPCK trypsin, α-chymotrypsin, V8 protease, aminopterine, bovine serum albumin, 5-bromodeoxyuridine (BrdU), calf thymus DNA, human hemoglobin, hypoxanthine, RNAse A, tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel) heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-Fluorenylmethylchloroformate (Fmoc-Cl), β-mercaptoethanol, L-cysteine, guanidine.HCl, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), N-Boc-1-*tert*-butyl-L-aspartate (Boc-Glu-OtBu) (Novabiochem); trifluoroacetic acid, thiodiglycol (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNAse T1 (Boehringer, Mannheim, Germany); penicillin (Gist Brocades, Delft, The Netherlands); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat, (Campina, Eindhoven, The Netherlands); sodium azide (BDH, Poole, UK); streptomycin (Biochemie, Vienna, Austria); and [^{14}C]bromoacetic acid (Amersham, Houten, The Netherlands).

Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands).
Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10kD filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA).
Human callus was obtained from chiropodists.

II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).

HPLC analyses were carried out by using a Waters model 510 HPLC pump and an Applied Biosystems 757 detector. The analyses were performed on a Lichrosorb reverse phase C18 column (250x5 mm), on a reversed-phase column Chromspher C-18 (100x3 mm, particle size 5 µm; Chrompack, Middelburg, The Netherlands) or on a Lichrosorb reverse phase RP-select B column (250x4 mm, particle size 5 µm; Merck, Darmstadt, Germany). Radiometric detection was performed with a Radiometric Flo-one/Beta A-500 radiochromatography detector (Canberra Packard) using Flo-Scint A (Canberra Packard) as a scintillation cocktail.

TLC was performed on Merck HPTLC plates (60F 254; 5x10 cm) or on Merck RP-18 plates (5x20 cm).
FPLC analyses were carried out on a PepRPC 5/5 column using two pumps P-500, a controller LCC-501 plus and a UV-M II monitor (all Pharmacia, Uppsala, Sweden).
Gel filtration on Sephadex G-75 and LH-20 (Pharmacia) was performed with a P-1 pump, GP-250 gradient programmer, Frac-100 fraction collector, a UV-1 optical unit (254 nm) and a UV-1 control unit (Pharmacia).

LC-MS-MS spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (Fisons, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 V, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C. The LC system comprised a reverse phase C18 column (Lichrosorb, 5 µm particles) with water/acetonitrile/formic acid (80/20/0.1, v/v/v) as an eluent. The flow rate was 0.8 ml/min with a split of ca. 1/10 to the mass spectrometer; the injection volume was 10-40 µl. A few analyses were performed with a Q-TOF MS (Micromass, Wythenshawe, U.K.), using a similar LC system.

GC-NCI/MS analyses were performed with a VG70-250S mass spectrometer (Fisons Instruments, Altrincham, U.K.) operated in the NCI mode (methane) with a source temperature of 200 °C, an ionization energy of 70 eV, and an ion source pressure of 2 mPa.
The gas chromatograph (HP 5890A) was equipped with an on-column injector (Carlo Erba, Milan, Italy) and a CPSil 5CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands). The oven of the chromatograph was kept at 120 °C for 5 min; the temperature was then programmed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min.
For thermodesorption/cold trap (TCT) injection, the sample (50 µl) was tranferred onto a clean Tenax tube (Chrompack). A helium flow (50 ml/min) was applied during 30 min in order to evaporate the solvent. Next, the Tenax tube was placed in a TCT unit (Chrompack) and rapidly heated to 250 °C while holding the cold trap at - 125 °C. The helium flow rate was 15 ml/min. After 10 min, the helium vent located directly behind the cold trap was closed resulting in a

helium flow rate through the analytical column of 1.5 ml/min and the cold trap was flash-heated to 250 °C.

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to tetramethyl silane. The solvent signals at 2.525 ppm (residual Me_2SO-d_5 in Me_2SO-d_6) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference for ^{1}H NMR spectroscopy, whereas the solvent signals at 39.6 ppm (Me_2SO-d_6) or 77.1 ppm ($CDCl_3$) were used as a reference for ^{13}C NMR spectroscopy.

Radioactivity counts were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.
Thin layer chromatograms of radioactive products were scanned using a Bioscan System 200A Imaging Scanner (Bioscan Inc., Washington, DC, U.S.A.).

Peptides were synthesized on an Abimed (Langenfeld, Germany) AMS 422 peptide synthesizer. Peptides were analyzed by FPLC using a reversed phase PEP-RPC 5/5 column. Linear gradient elution (1 ml/min) was performed from 0.1% TFA/H_2O to 0.1% TFA/70% CH_3CN in 20 min. Detection was at 214 nm.

SDS-PAGE on albumin was performed on a BioRad system, applying Coomassie brilliant blue R250 coloration.

Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). DNA was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). A Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The developed film was scanned with a densitometer (Ultroscan XL, Pharmacia). In later experiments the chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

III EXPERIMENTAL PROCEDURES

III.1 Development of immunochemical assays of sulfur mustard adducts to DNA as Standard Operating Procedures

III.1.1 Treatment of DNA with sulfur mustard

A solution of double-stranded calf thymus DNA (1 mg/ml) in TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.4) was treated with sulfur mustard in acetonitrile (0.01-10 µM; final acetonitril concentration 1%) at 37 °C for 30-60 min and subsequently stored at -20 °C. In alternative experiments, the DNA solution was mixed with sulfur mustard solution at room temperature and subsequently incubated in an incubator at 37 °C or at room temperature.

III.1.2 Treatment of human blood with sulfur mustard and isolation of blood cells

Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing Na_2EDTA (15 mg). The blood sample was treated with sulfur mustard in acetonitrile (0.01-10 µM; final acetonitril concentration 1%) at 37 °C for 30-60 min. In alternative experiments, blood was mixed with an appropriate sulfur mustard solution at room temperature and subsequently incubated in an incubator at 37 °C or at room temperature.

III.1.3 DNA isolation from human blood

Several DNA isolation procedures have been applied and are described below. In general, these procedures include the lysis of erythrocytes, the lysis of the white blood cells (WBC), sometimes combined with a treatment with proteinase K, an RNAse A and T1 treatment, protein precipitation, DNA precipitation, solvation of the DNA pellet, and measurement of the DNA concentration. The isolation was carried out on 300 µl of whole blood, except in the case of the phenol/chloroform/isoamylalcohol extraction method in which 1 ml of blood was used. In all cases, the methods have been applied to both frozen and fresh blood. After the final wash with 70% ethanol and drying on air or with a 'Speedvac', the DNA was resuspended in TE buffer. The DNA-concentration was determined spectrophotometrically (ε_{260nm} = 6,600 $l.mol^{-1}.cm^{-1}$, expressed per mol of nucleotide) in a 20-fold dilution of a 4-µl aliquot of the DNA solution, with an uncertainty of about 5% (standard deviation). The purity of the solution was checked by determining the A_{260}/A_{280} ratio of the DNA solution.

Isolation with phenol/chloroform/isoamylalcohol extraction

Lysis of the erythrocytes in blood (1 ml) was brought about by incubation of the cell suspension with three volumes of freshly prepared lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) at 0 °C for 5 min. After centrifugation for 15 min at 400 g (4 °C), the supernatant, containing the hemoglobin, was removed. The pelleted WBC were washed twice with PBS and resuspended in TE buffer (1 ml). Sodium dodecyl sulfate (SDS; final concentration 1%, w/v) was added to lyse the cells and proteinase K (final concentration 500 µg/ml) to digest the protein. The lysates were incubated overnight at 37 °C. DNA was purified by gently shaking with an equal volume of phenol, saturated with 1 M Tris-HCl, pH 8, for 15 min, followed by separation and removal of the phenol layer and two additional extractions with equal volumes of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) and chloroform/isoamylalcohol (24:1, v/v), respectively. After addition of 0.1 volume of 3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5, the DNA was precipitated with two volumes of absolute ethanol, pre-cooled at -20 °C. After centrifugation at 3000g for 3 min, the pellet was washed in 70% ethanol and dissolved overnight

in TE buffer (1 ml). Next, the solution was incubated with RNAse A (final concentration 75 µg/ml, heated at 80 °C for 5 min to destroy any DNAse activity) and RNAse T1 (final concentration 75 units/ml) at 37 °C for 2 h in order to digest the RNA and subsequently with proteinase K (100 µg/ml) for 1 h to digest protein. The DNA was purified by repeating the phenolic extraction procedure and alcohol precipitation as described above. The DNA was dissolved overnight in TE buffer (300 µl) under continuous vibration at room temperature.

Isolation by using a PureGene kit (Biozym)
RBC Lysis Solution (900 µl) was added to blood (300 µl) in order to lyse the red blood cells and the mixture was centrifuged at 14,000g for 20 s. The pelleted white blood cells were lysed with Cell Lysis Solution (300 µl). In some cases, proteinase K was added to a final concentration of 100 µg/ml. Lysis was achieved under continuous slow shaking on a rotating wheel at 37 °C until a clear solution was obtained, lasting about 2 h. Then, RNAse A treatment (1.5 µl; 50 µg/ml) was carried out for 15 min at 37 °C, followed by cooling to 20 °C and addition of Protein Precipitation Solution (100 µl). After centrifugation at 14,000g for 3 min, the supernatant was transferred to a tube containing isopropanol (300 µl) to precipitate the DNA. After centrifugation (14,000g for 3 min), the pellet was washed with 70% ethanol (300 µl), dried in a 'Speedvac' and dissolved overnight in TE buffer (100 µl) under continuous vibration at room temperature. To speed up the procedure the DNA pellet could also be dissolved by incubation at 65 °C for 30 min. However, the latter modification might result in some loss of N7-HETE-Gua from the DNA and lower DNA yields.

Isolation by using a Xtreme™ Genomic DNA Purification Kit (Pierce)
Reagent A (900 µl)was added to blood (300 µl) and the mixture was centrifuged (1300g, 5 min) after shaking at room temperature during 5 min. The supernatant was discarded, Reagent B (340 µl) was added and the mixture was vortexed briefly to resuspend the pellet and left overnight at room temperature. After addition of a RNAse A solution (2.5 µl; 50 µg/ml), the solution was incubated at 37 °C for 30 min. Then, 5 M Sodium Perchlorate Solution (100 µl) was added and the mixture was shaken at 37 °C for 20 min, followed by 20 min incubation at 65 °C. After cooling to room temperature, the DNA was extracted by adding chloroform (580 µl), shaking for 20 min at room temperature and centrifugation at 1300g for 1 min. Then, XtremeTM Silica suspension (45 µl) was added and the mixture was centrifuged at 1300g for 4 min. The DNA-containing phase was poured off and ethanol (880 µl) was added to precipitate the DNA. The DNA was centrifuged at 4000g for 5 min and washed with 70% ethanol (0.9 ml). The pellet was dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

Isolation by using a Pharmacia kit
Equal volumes (300 µl) of whole blood and ice-cold Cell Lysis Buffer were mixed gently and incubated on ice for 5 min and then centrifuged at 4000g for 1 min to pellet the nuclei. The supernatant was discarded and the pellet washed two times with Cell Lysis Buffer, 1:1 diluted with water (500 µl), by gently mixing and subsequent centrifugation. To the creamy white pellet, Extraction Buffer (50 µl) was added. The mixture was vortexed gently and left overnight at room temperature. Subsequent to a 10 min incubation period at 55 °C, Application Buffer (800 µl) was added and the mixture was incubated at room temperature for 5 min. Part of the supernatant (ca. 400 µl) was brought on a pre-spun MicroSpin Column and mixed. After 1 min, the column and the support tube were spinned at 735g for 2 min. The remaining half of the supernatant (400 µl) was added to the same pre-spun column and mixed. After 1 min, the column and the support tube were spinned at 735g for 2 min. After washing of the column with Wash Buffer (400 µl), the DNA was eluted from the column by adding twice Elution Buffer (200 µl), followed by centrifugation.

The DNA was precipitated by adding isopropanol (320 µl) to eluted DNA (400 µl) and leaving it for 10 min at room temperature, followed by centrifugation at 735g for 10 min. The pellet was washed with 70% ethanol (500 µl), dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

Isolation by using a Wizard™ Genomic DNA Purification Kit (Promega)
Whole blood (300 µl) was added to Cell Lysis Solution (900 µl) and gently mixed. After 10 min of incubation at room temperature to lyse the red blood cells, the suspension was centrifuged at 14,000g for 1 min. The supernatant was discarded and the pellet resuspended in the remaining supernatant. Then, Nuclei Lysis Solution (300 µl) was added and pipetted 5 times to lyse the WBC. The suspension was incubated at 37 °C until a clear solution was obtained (2 h). RNAse Solution (1.5 µl) was added to the nuclear lysate and the mixture was incubated at 37 °C for 15 min. After cooling to room temperature, Protein Precipitation Solution (100 µl) was added. The mixture was vortexed vigorously for 20 s and centrifuged at 14,000g for 3 min. The supernatant was transferred to a clean tube containing isopropanol (300 µl) at room temperature, mixed gently and centrifuged at 14,000g for 1 min. The pellet was washed with 70% ethanol (500 µl), air-dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

Isolation by using a Stratagene DNA MicroExtraction Kit (Westburg)
To Solution 1 (1.2 ml) blood (300 µl) was added and incubated on ice for 2 min. The nuclei were pelleted at 14,000g for 10 min, washed once with Solution 1 and centrifuged again. The pellet was resuspended in Solution 2 (330 µl) and pronase solution (1 µl; 225 mg/ml) was added. After incubation at 37 °C for 2 h, the mixture was chilled on ice for 10 min. Solution 3 (120 µl) was added to precipitate the protein. After centrifugation at 14,000g for 15 min, the supernatant was transferred to another tube, RNAse (1 µl; 10 mg/ml) was added and the mixture was incubated at 37 °C for 15 min. DNA was precipitated by addition of 2 volumes of 100% ethanol, cooled at -20 °C for 10 min and centrifuged at 14,000g for 5 min at 4 °C. The precipitate was washed with 70% ethanol, dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

Isolation by using a DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim)
To Red Blood Cell Lysis Buffer (900 µl) whole blood (300 µl) was added. After gently shaking for 10 min, the mixture was centrifuged at 14,000g for 20 s. The supernatant was discarded and the pellet was resuspended in the residual supernatant. White Cell Lysis Buffer (300 µl) was added and mixed thoroughly by vortexing. After a clear solution was obtained (45 min at 37 °C), RNAse A (1.5 µl; 50 µg/ml) was added to a final concentration of 0.02 µg/ml and incubated at 37 °C for 15 min. After cooling to room temperature, Protein Precipitation Solution (150 µl) was added. The mixture was vortexed thoroughly and then centrifuged at 12,000g for 10 min. The supernatant was poured carefully into another tube and 2 volumes ethanol were added at room temperature, gently mixed and centrifuged at 12,000g for 10 min. The pellet was washed with 70% ethanol (1 ml), dried at 37 °C for 15 min and dissolved overnight under continuous vibration at room temperature in TE buffer (100 µl).

III.1.4 DNA isolation from human skin

DNA from human skin was isolated by separating the epidermis from the dermis by dispase treatment overnight, lysis of the epidermal layer and further following the same procedure as for blood after lysis of the red blood cells and pelleting the WBC (see Subsection III.1.3). To this purpose skin biopsies (about 3x3 mm) were first treated overnight at 4 °C in a 3-cm petri-dish with the enzyme dispase (2.4 mg/ml PBS; 3 ml) to separate the epidermis from the dermis (by

layering the pieces of skin on the dispase solution and shortly emersing the pieces in it). The epidermis was transferred to an Eppendorf tube and then the lysis solution was added, followed by RNAse A (3 µl of 75 µg/ml) treatment at 37 °C for 1 h. The procedure was continued as described for isolation of DNA from the blood samples by using a PureGene kit of Biozym.

III.1.5 DNA denaturation

Single- and double-stranded calf thymus DNA or DNA from sulfur mustard-exposed human white blood cells were made single-stranded by thermal denaturation in TE-buffer containing 4.1% formamide and 0.1% formaldehyde (50 µg DNA/ml) at 52 °C for 15 min, followed by rapid cooling on ice and storage at -20 °C.

III.1.6 Immunoslotblot procedure for N7-HETE-Gua

Several modifications were applied to the previously described procedure (7). So far, the following procedure appeared to be the optimum method. In the immunoslotblot assay (ISB) the single-stranded DNA containing N7-HETE-Gua was first slotblotted onto a nitrocellulose filter. Thermally denatured DNA was diluted in PBS to a final concentration of 5 µg/ml. The solution (200 µl) was spotted on a nitrocellulose filter. Ten positions on the 96-blots filter were occupied by calibration samples of DNA with adduct levels in the range of 0-10 N7-HETE-Gua/10^7 nucleotides. All samples were blotted in duplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the DNA was immobilized by UV crosslinking (50 mJ/cm^2). The next steps in the procedure, treatment with blocking solution, 1st antibody (2F8, directed against N7-HETE-Gua in DNA) and 2nd antibody (rabbit-anti-mouse-Ig-horse radish peroxidase), were the same as described previously (7). The solutions A and B of the chemiluminescence blotting detection system were mixed (100:1) and equilibrated for 1 h at 25 °C before addition to the filter. The filters were incubated for 1 min in substrate and then placed in a plastic bag. Excess of liquid was pressed out. Next, the filters were placed in a luminometer and the chemiluminescence was measured.

III.2 Development of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin as a Standard Operating Procedure

III.2.1 Synthesis of [^{14}C]sulfur mustard

To a solution of [^{14}C]bromoacetic acid (spec. act. 52 mCi/mmol; 0.5 mmol) in THF was added borane tetrahydrofuran complex solution (0.75 mmol; 1.0 M). The reaction was performed in a 4 ml vial under cooling in an ice bath. After 16 h at room temperature the reaction mixture was quenched by the addition of H_2O (15 µl) and added to a mixture of ethanol (0.5 ml), β-mercaptoethanol (42 µl) and NaOEt solution (21% in ethanol; 225 µl). After 45 min, 2.25 h and 3.75 h, an extra portion (50 µl) of NaOEt solution was added. The mixture was stored overnight at -20 °C. TLC analysis (8% methanol in CH_2Cl_2) with radiometric detection showed the presence of radioactive material that coeluted with thiodiglycol. Acetic acid (20 µl) was added and the mixture was concentrated under reduced pressure. The crude [^{14}C]thiodiglycol was purified by means of silica gel column chromatography. Elution was performed with a gradient of 0-5% methanol in CH_2Cl_2 (50 ml). Fractions were checked with TLC (eluent 6% methanol in CH_2Cl_2), vy menas of detection with I_2 vapour and scanning for radioactivity. The main impurity (the disulfide of β-mercaptoethanol), having a slightly larger R_f value, did not contain radioactivity. The fractions containing pure [^{14}C]thiodiglycol were collected, concentrated and coevaporated with $CHCl_3$ for removal of methanol. Fractions containing [^{14}C]thiodiglycol contaminated with the disulfide were also collected, concentrated and re-chromatographed. The

total yield of [^{14}C]thiodiglycol was determined with liquid scintillation counting: 14 mCi, spec. act. 52 mCi/mmol (0.27 mmol, 54%).
The purified [^{14}C]thiodiglycol was dissolved in $CHCl_3$ (2 ml) and thionyl chloride (96.4 mg; 0.81 mmol; 60 µmol) was added under cooling in an ice bath. The mixture was heated under reflux for 3 h. GC-analysis showed, in addition to the presence of a peak with the same retention time as sulfur mustard, an impurity with a longer retention time. The crude product was diluted with cold sulfur mustard (0.2 mmol; 32 mg) and fractionated by distillation under reduced pressure, as described earlier (7). The fractions were analyzed by gas chromatography. Peaks were collected in Carbosorb and radioactivity was determined after addition of Permablend scintillation cocktail. The purity was established from TLC analysis. Two batches of pure [^{14}C]sulfur mustard resulted:
batch 1: 23 mg, spec. act. 15 mCi/mmol, radiochemical purity 99+%
batch 2: 9 mg, spec. act. 15 mCi/mmol, radiochemical purity 99+%
One of the impure fractions was combined with an older batch of impure [^{14}C]sulfur mustard and redistilled:
batch 3: 18 mg, spec. act. 14 mCi/mmol, radiochemical purity 97%

III.2.2 Incubation of human blood with sulfur mustard, [^{14}C]sulfur mustard or sulfur mustard-d_8

A 1 M solution of sulfur mustard, [^{14}C]sulfur mustard (sp. act. 15 mCi/mmol) or sulfur mustard-d_8 in CH_3CN was prepared. For an exposure level of 10 mM, 50 µl of this solution or of an appropriate dilution in CH_3CN was added to human blood (5 ml). After incubation at 37 °C, plasma and erythrocytes were separated by centrifugation at 3,000 rpm.

III.2.3 Isolation of globin from human blood

Globin was isolated from human blood samples according to Bailey *et al.* (15). The red blood cells were washed four times with saline and lysed with water. After 30 min in ice/water, they were centrifuged for 30 min at 25,000g (4 °C). The supernatant was poured into a stirred mixture of concentrated HCl/acetone (1/100, v/v) at -20 °C. After decanting the supernatant, the formed precipitate was washed with concentrated HCl/acetone (1/100, v/v), acetone and ether, and dried. For some experiments, the crude globin was purified via a G-25 Sephadex column, using 0.1 M formic acid, 6 M urea and 50 mM dithiothreithol as an eluent. UV-positive fractions were pooled and dialyzed three times against a 1 mM phosphate buffer, pH 7. Finally, the globin was dialyzed against water for 2 h and lyophilized to give a white fluffy compound.

III.2.4 Original procedure for modified Edman degradation of globin

Globin (20 mg, originating from human or guinea pig blood exposed to sulfur mustard) was dissolved in formamide (2 ml). Pyridine (6 µl) and PFPITC (6 µl) were added. The mixture was incubated overnight at room temperature followed by 2 h at 45 °C. The formamide layer was extracted with diethyl ether (3 × 1.5 ml). The combined ether fractions were dried under a stream of nitrogen and the resulting residue was dissolved in toluene (1 ml). The toluene solution was washed, dried, and concentrated to a small volume (500 µl). Heptafluorobutyrylimidazole (10 µl) was added and the mixture was heated for 10 min at 45 °C. After washing with water (4 × 0.5 ml), the organic layer was dried ($MgSO_4$) and concentrated. The residue was dissolved in 100 µl toluene and analyzed with GC/MS.

III.2.5 Simplified procedure for modified Edman degradation of globin

A mixture of globin (20 mg), PFPITC (6 µl) and pyridine (6 µl) in formamide (2 ml) was heated for 2 h at 60 °C. Subsequently, the mixture was extracted with toluene (3 × 0.5 ml). Separation of the toluene/formamide layers was achieved by freezing in liquid nitrogen. The toluene layers were washed consecutively with water (2 × 0.5 ml), aqueous Na_2CO_3 (0.1 M, 0.5 ml) and water (0.5 ml) and concentrated to a small volume (500 µl). Heptafluorobutyrylimidazole (10 µl) was added and the mixture was heated for 10 min at 45 °C. After washing with water (4 × 0.5 ml), the organic layer was dried ($MgSO_4$) and concentrated. The residue was dissolved in toluene (100 µl) and analyzed with GC/MS.

III.3 Detection of hemoglobin adducts

III.3.1 Synthesis of Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine

Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester (103 mg; 0.25 mmol), which was synthesized as described previously (7), was dissolved in dry HCl/ethyl acetate (1 M; 62 ml). After stirring for 3 h at room temperature, FPLC analysis showed complete conversion into a single compound with a shorter retention time. The solution was concentrated under reduced pressure. Subsequently, the residue was dissolved in methanol/water (9/1, v/v; 4.5 ml) containing 0.2 M NaOH. After 1 h, FPLC analysis showed complete conversion into a compound with a shorter retention time. The reaction mixture was neutralized with acetic acid (50 µl) and concentrated. The residue was dissolved in a mixture of dioxane and aqueous 10% Na_2CO_3 (1/2, v/v; 6 ml). The solution was stirred under cooling in an ice-bath and subsequently a solution of Fmoc-Cl (86 mg; 0.33 mmol) in dioxane (2 ml) was added dropwise. After stirring for 16 h at room temperature, the mixture was diluted with water (10 ml) in order to dissolve solid material and extracted with pentane (5 × 15 ml) to remove unreacted and hydrolyzed Fmoc-Cl. The aqueous layer was acidified with 20% acetic acid to pH 3.5 and extracted with CH_2Cl_2 (3 × 20 ml). The organic layer was dried ($MgSO_4$) and concentrated, giving a white foam (95 mg; 70% over three steps, i.e., removal of Boc group, saponification, introduction of Fmoc group).
^{1}H-NMR ($CDCl_3$): δ 8.0 - 7.0 (m, 10H, H-arom., H-imid.), 4.5 - 4.0 (m, 6H, NCH_2, CH-α, OCH_2-CH), 3.5 (m, 2H, CH_2-OtBu), 3.1-3.2 (m, 2H, CH_2-β), 2.9 (m, 2H, CH_2S), 2.6 (m, 2H, CH_2S), 1.2 (m, 9H, tBu).
^{13}C-NMR ($CDCl_3$): 174.9 (COOH), 155.7 (NC=O), 144 - 120 (C-arom., C-imid.), 73.5 [$\underline{C}(CH_3)_3$], 66.6 ($\underline{C}H_2$-OCO), 62.4 (CH_2O-tBu), 53.9 (N-$\underline{C}$H-CH_2), 48.0 (N-CH_2), 34 - 33 (2× CH_2S).
Electrospray MS: *m/z* 538 (MH^+), 482 ($MH^+ - C_4H_8$), 161 (H_2C=CH-S-CH_2-CH_2-O-tBu).

III.3.2 Synthesis of peptides containing a N1/N3-HETE-histidine moiety

The following peptides were synthesized:
1. A-F-S-D-G-L-A-H(N1/N3-HETE)-L-D-N-L-K, which represents the amino acid residues 70-82 of human β-globin
2. G-K-V-G-A-H(N1/N3-HETE)-A-G-E-Y-G-A-K, which represents the amino acid residues 15-26 (+ lysine) of human α-globin
3. L-H(N1/N3-HETE)-V-D-P-E-N-F-R-L-L-G-N-V-K, which represents the amino acid residues 96-109 (+ lysine) of human β-globin.

The synthesis was carried out on a 10 µmol scale with an automated solid phase peptide synthesizer. Before introduction of the modified residue, the synthesis was stopped and a solution of Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine in NMP (0.27 mg/µl; 110

µl) was added to the resin, together with a solution of PyBOP in NMP (0.54 mg/µl; 60 µl) and a solution of NMM in NMP (40 µl NMM in 175 µl NMP; 60 µl). Subsequently, the synthesis was continued as described earlier. FPLC analysis showed the presence of one main product in each case.
Electrospray MS analysis showed the presence of the expected mass:
1. *m/z* 753.3 (MH_2^{2+}), 502.8 (MH_3^{3+})
2. *m/z* 675.2 (MH_2^{2+}), 450.8 (MH_3^{3+})
3. *m/z* 876.4 (MH_2^{2+}), 584.9 (MH_3^{3+}), 439.1 (MH_4^{4+})

Furthermore, the sequence of the peptides was firmly established by means of tandem MS analysis.

III.3.3 Immunization of mice for generation of antibodies against haptens synthesized[2]

For each hapten three mice were immunized (i.p.) with 50 µg of antigen to which spekol was added (5-10 ml/kg). Blood samples of all mice were taken after 7 days to test the serum for antibody response against hemoglobin or keratin treated with 50 µM sulfur mustard, with a direct ELISA (see Subsection III.3.6). A positive response was not observed against sulfur mustard treated proteins after 7 days. Therefore, the mice received a second immunization with the same hapten at 4 weeks after the first immunization. After the second immunization still no positive response was observed against sulfur mustard treated proteins or the antigen itself. Nevertheless, a booster with antigen (volume up to 0.2 ml) was administered 4-12 weeks later. After 3 days the animals with the strongest immune response against sulfur mustard treated protein or the antigen itself were killed with CO_2 anesthesia and the blood was collected by heart punction. A cell suspension of the spleen was prepared for the production of hybrid cell strains.

III.3.4 Production of hybrid cell strains

The spleen cells of the mouse were isolated for fusion with SP2/0 plasmacytoma cells. The SP2/0 plasmacytoma cells were grown in RPMI1640-medium supplemented with 10% FCS, 1 mM sodium pyruvate, 1 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 mM ß-mercaptoethanol. Spleen cells and SP2/0 cells were washed twice in RPMI-medium without serum. Next, 10^8 spleen cells were added to 10^7 SP2/0 cells and centrifuged (20 min at 50*g*). The supernatant was removed and the cells were exposed to fusion conditions by brief consecutive incubations of a mixture of these cells in 41% and 25% PEG 4000 as follows. The cell pellets were resuspended for 1 min in 41% PEG-solution (0.5 ml). Then, 25% PEG-solution (0.5 ml) was added and the mixture was shaken slowly for 1 min. RPMI-medium without serum (4 ml) was added twice and the cell suspension was shaken slowly for 2 min. The cell suspension was incubated for 15-30 min at room temperature and then centrifuged (20 min at 50*g*). The supernatant was removed and the pellet resuspended in RPMI-medium with 10% FCS. The cells were seeded in a 75-cm^2 culture flask and incubated overnight. After 24 h of incubation, the cells were centrifuged (20 min at 10*g*) and the cells were resuspended in complete RPMI-medium (38 ml; the same medium as used for growing of SP2/0 cells) supplemented with HAT-medium, i.e., 0.1 mM hypoxanthine, 16 mM thymidine, and 0.4 mM aminopterine. Hybridomas were selected in HAT-medium because they can grow in this medium whereas SP2/0 cells do not survive; spleen cells cannot be cultured (16). The cells were seeded in 96-well polystyrene culture plates in HAT-medium. Hybrid cells were cultured and refreshed in this selective HAT-medium and their supernatants were screened for specific antibody production in a direct ELISA (as described in

[2] The procedures described in subsections III.4.3 - III.4.6 were also used for generation of antibodies against haptens which were derived from sulfur mustard adducts with keratin.

Subsection III.3.6). Cells producing specific antibodies against sulfur mustard treated proteins were recloned twice by limiting dilution as will be described in the next subsection.

III.3.5 Cloning of hybridomas by limiting dilution

Cells of the fusion mixture producing specific antibodies against sulfur mustard treated protein (hemoglobin or keratin) were counted by light-microscopy and diluted in HAT-medium to a concentration of 50, 10 and 5 cells/ml. Per well of 96-well culture plates, 0.1 ml of one of these solutions was added resulting in 5, 1 and 0.5 cell/well. The plates were incubated for eight days without refreshing the medium. Subsequently, the amount of clones per well was counted. The supernatants of wells with only one clone were tested for specific antibody activity against sulfur mustard treated hemoglobin. Clones showing a positive result were recloned once again by limiting dilution to make sure that monoclonal antibodies would be obtained.

III.3.6 Immunoassays (ELISA) with the polyclonal antisera and hybridoma-supernatants.

The polyclonal antisera and hybridoma-supernatants were tested in a direct ELISA against hemoglobin treated with sulfur mustard (0, 50, 100, 500 µM), globin isolated from sulfur mustard-treated hemoglobin, and against the immunogen itself (if available in sufficient amounts). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted hemoglobin, keratin or peptides dissolved in water to a final concentration of 10 µg/ml or with adducted and non-adducted globin dissolved in water to a final concentration of 2.5 µg/ml. Of these dilutions 50 µl was added per well and incubated overnight at 37 °C. The plates were washed three times with PBS containing 0.05% Tween 20. Next, the plates were incubated with PBS containing 1% FCS for 60 min at 37 °C and again washed three times with PBS containing 0.05% Tween 20. The polyclonal antisera and the hybridoma supernatants were diluted 10-1,000 times and 5-100 times, respectively, in PBS with 0.05% Tween 20 and 0.1% FCS. Of these dilutions 50 µl was added per well and incubated for 60 min at 37 °C. After washing, the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 1:1,000 in PBS containing 0.05% Tween 20, 0.5% gelatin, and 5% FCS, was added (50 µl/well) and the plates were incubated for 60 min at 37 °C. After three washings with PBS containing 0.05% Tween 20, the plates were washed once with 0.1 M diethanolamine, pH 9.8 (100 µl). A solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 1 h.

III.4 Detection of albumin adducts

III.4.1 Isolation of albumin from plasma

Albumin was isolated from human plasma according to a procedure described by Bechtold et al. (17). Thus, shortly, whole blood was collected into an EDTA-containing vacutainer and separated into red blood cells and plasma. To the plasma an equal volume of 0.5 M $CaCl_2$ was added. The mixture was incubated at room temperature overnight and then centrifuged at 900 g for 20 min. To the supernatant were added 4 volumes of 0.9% saline. Nine volumes of an acid/alcohol mixture (made by adding 1 ml 12 M HCl to 600 ml ethanol) were added dropwise to the supernatant. The mixture was incubated at 37 °C for 30 min and then centrifuged at 650 g for 5 min. To the supernatant was added a volume of 0.2 M sodium acetate in 95% ethanol equal to 1/10 the total volume of the supernatant. After 15 min the mixture was centrifuged at 650 g for 5 min, the supernatant discarded, and the albumin pellet washed with acetone. The

mixture was centrifuged at 650 g for 5 min and the supernatant discarded. The pellet was then washed in diethyl ether, centrifuged and allowed to dry overnight. Yields: 50-60 mg/ml plasma. Analysis with SDS PAGE showed coelution with commercially available human serum albumin.

III.4.2 Tryptic digestion of albumin

Prior to tryptic digestion the disulfide bridges were reduced with dithiothreitol and the resulting thiol functions were carboxymethylated. To a solution of albumin (3 mg) in a buffer (300 µl) containing 6 M guanidine.HCl, 100 mM Tris.HCl and 1 mM EDTA, pH 8.3 (with 2 M NaOH), dithiothreitol (5 mg) was added and the solution was incubated at 55 °C for 40 min. Subsequently, iodoacetic acid (sodium salt; 10 mg) was added and the mixture incubated at 40 °C for 30 min. The clear solution was transferred into a Slide-a-Lyzer cassette (0.1-0.5 ml) and the solution dialyzed against aqueous NH_4HCO_3 (3 l) for 16 h. Trypsin (2% w/w) was added and the mixture was incubated at 37 °C for 4 h. Albumin samples isolated from human blood according to the procedure described in Subsection III.4.1 gave similar HPLC chromatograms after tryptic digestion when compared to a commercially available albumin sample.

III.4.3 Synthesis of the sulfur mustard adduct of T5 of albumin

To a solution of S-HETE-cysteine (1 mmol; 225 mg) in dioxane/water (5 ml; 1/1, v/v) was added Fmoc-Cl (1 mmol; 260 mg) and Na_2CO_3 (270 mg) under stirring at 0°C. After 4 h, stirring was continued at room temperature for 16 h. The solution was washed with petroleum ether 60-80 and the aqueous layer was acidified (pH 3) with 1 M $KHSO_4$ (20 ml). The aqueous layer was extracted with ethyl acetate (2 × 20 ml). The organic layers were collected, dried ($MgSO_4$) and concentrated, giving a colourless oil (400 mg; 89%). FPLC analysis showed the presence of one main compound. This compound was used without further purification for the solid phase synthesis of the sulfur mustard adduct of the T5 tryptic fragment of albumin, i.e., A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C(S-HETE)-P-F-E-D-H-V-K. After splitting from the resin one main compound resulted according to FPLC analysis. This compound was used for immunochemical experiments and as reference for tandem MS experiments and HPLC analyses.
Electrospray MS: *m/z* 1269.8 (MH_2^{2+}), 847.0 (MH_3^{3+}), 635.5 (MH_4^{4+}).

III.4.4 LC-tandem MS analyses in tryptic digests of albumin

A PRP-1 column (length 25 cm; i.d. 0.3 mm) was used in the LC system. Eluent A consisted of water/acetonitrile, 95/5, containing 0.5% formic acid and eluent B consisted of water/acetonitrile, 2/8, containing 0.5% formic acid. The following flow scheme was applied: 100% eluent A at a flow of 0.1 ml/min from 0-5 min and, subsequently, 100% eluent A to 100% eluent B at a flow of 0.3 ml/min from 5-90 min. Flow rates were reduced by a pre-injector split: 3 to 10 µl/min from 0-5 min and, subsequently, constant at 10 µl/min. The LC column was directly connected to the electrospray probe. The injection volume was 10-40 µl. Analyses with the VG triple quadrupole mass spectrometer were performed in the multiple reaction monitoring (MRM) mode (transition $MH_3^{3+} \rightarrow$ *m/z* 1071.0, 1014.5 and 978.5). Operating conditions were: cone voltage 35 V, collision energy 12 eV, argon pressure 5×10^{-3} mB, dwell 1.5 s/channel, span *m/z* 0.2, resolution MS1 and MS2 10. Full scan MS-MS spectra were acquired with the Q-TOF-MS.

III.5 Detection of keratin adducts

III.5.1 Isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residu was stirred in a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residu was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M).
The crude keratin was purified on a G 75 column (100 × 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized. Representative yield: 20 mg keratin/100 mg callus.

III.5.2 Exposure of human callus to [^{14}C]sulfur mustard

To a suspension of human callus (70-100 mg) in 0.9% NaCl (100 µl) was added a solution of an appropriate concentration of [^{14}C]sulfur mustard in isopropanol (100 µl). The mixture was incubated for 6 h at 37°C. Isolation of keratin was performed as described in Subsection III.5.1.

III.5.3 Synthesis of bis-O,O-pentafluorobenzoylthiodiglycol

To a solution of thiodiglycol (0.59 mg, 0.5 µl, 4.8 µmol) in a mixture of toluene and pyridin (9/1, v/v; 500 µl) was added pentafluorobenzoyl chloride (10 µl). The mixture was incubated at 45 °C for 10 min. Next, the mixture was washed with aqueous 5% $NaHCO_3$ (200 µl) and water (200 µl) and the organic layer was dried over $MgSO_4$.
GC/MS (EI^+): *m/z* 298 [M^+ - F_5C_6COOH], 239 [M^+ - $F_5C_6COOCH_2CH_2S$], 195 [$F_5C_6CO^+$].

III.5.4 Isolation and derivatization of thiodiglycol after alkaline hydrolysis of keratin

Purified keratin that was exposed to sulfur mustard or [^{14}C]sulfur mustard was incubated for 1 h at room temperature in aqueous NaOH (5 mg keratin/300 µl of 0.5 M NaOH). After neutralization with aqueous acetic acid, liberated thiodiglycol was isolated from the NaOH treated keratin sample by ultrafiltration over Centrex UF-2 filters (molecular cut-off 10 kDa). The filtrate was evaporated to dryness and the residue was coevaporated with toluene. Next, the residue was diluted with toluene (440 µl) and pyridin (50 µl) and pentafluorobenzoyl chloride (10 µl) was added. After 5 min at room temperature the mixture was filtrated over glass wool and analyzed by HPLC with radiometric detection.

III.5.5 Synthesis of Nα-Boc-Nω-HETE-glutamine 1-*tert*-butylester

To a solution of Boc-Glu-OtBu (0.30 g, 1 mmol) in NMP (5 ml) was added PyBOP (0.57 g; 1.1 mmol) and NMM (120 µl; 1.1 mmol). Subsequently, a solution of 2-(2-aminoethylthio)ethanol (0.13 g, 1.1 mmol) in NMP (2 ml) was added. The reaction mixture was stirred for 4.5 h at room temperature. The mixture was taken up in CH_2Cl_2 (20 ml) and washed with 10% aqueous $NaHCO_3$ (3 × 10 ml), 0.1 M $KHSO_4$ (pH 5.3) and water (3 × 25 ml). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude compound was purified by means of silica gel column chromatography (eluent gradient: methanol/CH_2Cl_2, 0/100 to 5/95, v/v). Fractions were analyzed with TLC (eluent: methanol/CH_2Cl_2, 16/84, v/v). The appropriate fractions were collected and concentrated to afford a light yellow oil. Yield: 0.142 g (34.9%).

^{1}H-NMR ($CDCl_3$): δ 1.37/1.39 [2× s, 18H, 2× C(CH_3)$_3$], 1.82/2.07 [m, 2H, CH-CH_2(βGlu)], 2.21 [t, 2H, $J_{H,CH2}$ = 6.74 Hz, CH_2-CH_2(γGlu)], 2.63/2.67 (2× t, 2× 2H, $J_{H,CH2}$ = 6.15 Hz, 2× S-CH_2), 3.39 (t, 2H, $J_{H,CH2}$ = 5.95 Hz, NH-CH_2), 3.68 (q, 2H, $J_{H,OH}$ = 5.76 Hz, CH_2-OH), 4.08 [*b*s, H, NH-CH(αGlu)], 5.35 (d, H, $J_{H,CH}$ = 8.13 Hz, NH-CH), 6.91 [*b*s, H, C(O)NH-CH2].
^{13}C NMR ($CDCl_3$): δ 27.9 [6× C(CH_3)$_3$], 31.8 (S-CH_2), 32.5 [CH-CH_2(βGlu)], 34.9 (S-CH_2), 39.0 (NH-CH_2), 46.2 [CH_2-CH_2(γGlu)], 53.6 [NH-CH(αGlu)], 61.0 (CH_2-OH), 79.9/82.1 [2× C(CH_3)$_3$], 155.9 [C(O)-NH-CH_2], 171.4/172.5 [2× C(CH_3)$_3$OC(O)].

III.5.6 Synthesis of Nα-Fmoc-Nω-HETE-glutamine

Nα-Boc-Nω-HETE-Glu-OtBu (0.142 g, 0.35 mmol) was dissolved in a mixture of TFA/water (95/5, v/v; 2 ml). After 2 h the mixture was concentrated and coevaporated with water. The residue was dissolved in a mixture of 10% aqueous Na_2CO_3 (2 ml) and dioxane (5 ml). Fmoc-Cl (0.118 g; 0.46 mmol was added in small portions and the mixture was stirred for 18 h at room temperature, after which the mixture was taken up in water. The aqueous mixture was washed with light petroleum (3 × 25 ml) to remove Fmoc-OH and excess Fmoc-Cl. The aqueous layer was acidified to pH 3.5 with 1 M $KHSO_4$ and extracted with ethyl acetate (3 × 25 ml). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude compound was purified with a Sephadex LH-20 column (eluent: CH_2Cl_2/methanol, 2/1, v/v). Fractions were checked with TLC (eluent: CH_2Cl_2/methanol/acetic acid, 45/4/1, v/v/v). The appropriate fractions were collected and concentrated to afford a white foam. Yield: 93 mg (56%).
^{1}H-NMR ($CDCl_3$): δ 1.99 [m, 2H, CH-CH_2(βGlu)], 2.20 [m, 2H, CH_2-CH_2(γGlu)], 2.64 (2× t, 2× 2H, $J_{H,CH2}$ = 5.95 Hz, 2× S-CH_2), 3.40 (t, 2H, NH-CH_2), 3.67 (t, 2H, CH_2-OH), 4.13 [t, H, $J_{H,CH2}$ = 6.84 Hz, CH-CH_2(Fmoc)], 4.32 [d, 2H, $J_{H,CH2}$ = 6.94 Hz, CH-CH_2(Fmoc)], 6.29 [d, H, NH-CH(αGlu)], 7.10 (*b*s, H, NH-CH_2), 7.23 [t, 2H, ($J_{H,C1H}$ = $J_{H,C8H}$)≈($J_{H,C3H}$ =$J_{H,C6H}$) = 7.54 Hz, $J_{H,C4H}$ =$J_{H,C5H}$ = 1.09 Hz, C2H and C7H], 7.32 [t, 2H, ($J_{H,C2H}$ =$J_{H,C7H}$)≈($J_{H,C4H}$ = $J_{H,C5H}$) = 7.54 Hz, C3H and C6H], 7.54 (t, 2H, $J_{H,C2H}$ = $J_{H,C7H}$ = 7.44 Hz, C1H and C8H), 7.69 (d, 2H, $J_{H,C3H}$ = $J_{H,C6H}$ = 7.64 Hz, C4H and C5H).

III.5.7 Synthesis of Nα-Boc-Nω-HETE-asparagine 1-*tert*-butylester

This compound was synthesized as described for the corresponding glutamine derivative (see Subsection III.5.6), starting with Boc-Asp-OtBu. Yield: 0.19 g (50%).
^{1}H-NMR ($CDCl_3$): δ 1.44/1.47 [2× s, 18H, 2× C(CH_3)$_3$], 2.69/2.74 (2× t, 2× 2H, $J_{H,CH2}$ = 6.34 Hz, 2× S-CH_2), 2.84 [t, 2H, CH-CH_2(βAsp)], 3.06 (*b*s, H, CH_2-OH), 3.44 (q, 2H, $J_{H,CH2}$ = 6.05 Hz, NH-CH_2), 3.75 (q, 2H, $J_{H,OH}$ = 5.85 Hz, CH_2-OH), 4.38 [m, H, NH-CH(αAsp)], 5.72 (d, H, $J_{H,CH}$ = 6.74 Hz, NH-CH), 6.62 [*b*t, H, $J_{H,CH2}$ = 5.55 Hz, C(O)NH-CH_2].
^{13}C-NMR ($CDCl_3$): δ 27.9 [6× C(CH_3)$_3$], 32.0/35.2 (2× S-CH_2), 38.4 [NH-CH(αAsp)], 39.1 (NH-CH_2), 51.2 [CH-CH_2(βAsp)], 61.1 (CH_2-OH), 79.9/82.1 [2× C(CH_3)$_3$], 155.8 [C(O)-NH-CH_2], 170.2/170.5 [2× C(CH_3)$_3$OC(O)].

III.5.8 Synthesis of Nα-Fmoc-Nω-HETE-asparagine

This compound was synthesized as described for the corresponding glutamine derivative (see Subsection III.5.6), starting with Nα-Boc-Nω-HETE-asparagine 1-*tert*-butylester. Yield: 0.113 g (51%).
^{1}H-NMR ($CDCl_3$): δ 2.67/2.69 (2× t, 2× 2H, $J_{H,CH2}$ = 6.05 Hz, 2× S-CH_2), 2.75/2.90 [m, 2H, CH-CH_2(βAsp)], 3.40 (t, 2H, NH-CH_2), 3.71 (t, 2H, $J_{H,OH}$ = 6.05 Hz, CH_2-OH), 4.22 [t, H, $J_{H,CH2}$ = 6.85 Hz, CH-CH_2(Fmoc)], 4.37 [m, 2H, CH-CH_2(Fmoc)], 4.51 [t, H, NH-CH(αAsp)], 7.31 [t, 2H, ($J_{H,C1H}$ = $J_{H,C8H}$)≈($J_{H,C3H}$ = $J_{H,C6H}$) = 7.53 Hz, $J_{H,C4H}$ = $J_{H,C5H}$ = 1.0 Hz, C2H and C7H), 7.40 [t, 2H, ($J_{H,C3H}$ = $J_{H,C7H}$)≈($J_{H,C4H}$ = $J_{H,C5H}$) = 7.54 Hz, C3H and C6H), 7.61 (t, 2H,

$J_{H,C2H} = J_{H,C7H}$ = 6.65 Hz, C1H and C8H), 7.75 (d, 2H, $J_{H,C3H} = J_{H,C6H}$ = 7.54 Hz, C4H and C5H).
^{13}C-NMR ($CDCl_3$): δ 31.7/34.9 (2× S-CH_2), 37.7 [NH-CH(αAsp)], 39.1 (NH-CH_2), 47.1 [CH-CH_2(Fmoc)], 51.6 [CH-CH_2(βAsp)], 61.1 (CH_2-OH), 67.2 [CH-CH_2(Fmoc)], 120.0/125.1/127.1/127.7 (8× CH-arom.), 141.3/143.8 (4× C-q) 155.8 [C(O)-NH-CH_2], 170.9 [CH_2OC(O)NH], 172.9 [C(O)OH].

III.5.9 Solid phase synthesis of peptides containing an Nω-HETE-glutamine or Nω-HETE-asparagine residue

The following peptides containing modified asparagine or glutamine residues were synthesized as described earlier for the peptides containing a modified histidine residue (see Subsection III.3.2):

1. G-V-V-S-T-H-Q(Nω-HETE)-Q-V-L-R-T-K-N-K
2. G-I-Q-Q(Nω-HETE)-V-T-V-N-Q-S-L-L-T-P-L-N-K
3. G-V-M-N(Nω-HETE)-V-H-D-G-K-V-V-S-T-H-E-K

Electrospray MS analysis:

1. *m/z* 1797.2 (MH^+)
2. *m/z* 1955.2 (MH^+)
3. *m/z* 1839.0 (MH^+)

IV RESULTS

IV.1 Development of immunochemical assays of sulfur mustard adducts to DNA as Standard Operation Procedure

IV.1.1 Introduction

The primary aim of this study is to develop a standard operating procedure for use in the appropriate environment, *i.e.*, immunoslotblot assay of sulfur mustard adducts to DNA in human blood and skin. To this purpose the various steps involved in the immunochemical assay have been simplified and mimimized as much as possible for application under field conditions, in analogy with our research on a biological radiation dosimeter (18,19). It seems worthwhile to develop 2 modes of standard operating procedures, one in which experimental time is as short as possible and the other one in which sensitivity is the most important factor. The modifications described in the next subsections are meant to simplify and to speed up the procedure while maintaining maximum sensitivity. In a later stage some modifications will been introduced to speed up the procedure accepting some decrease in sensitivity and accuracy.

IV.1.2 Isolation of DNA from WBC and skin biopsies

Sofar, DNA was isolated from WBC and skin biopsies as described in the final report of a previous grant (7). Briefly, WBC from blood, isolated after lysis of the erythrocytes, were lysed with 1% SDS, followed by extraction with phenol, phenol/chloroform and chloroform/isoamyl alcohol, ethanol precipitation, RNAse treatment, treatment with proteinase K, and again the same phenol/chloroform/isoamylalcohol extraction procedure followed by ethanol precipitation. The DNA concentration was determined in a 20-fold dilution of a 4-µl aliquot of the DNA solution with an uncertainty of about 5% (standard deviation).

DNA from human skin was isolated by separating the epidermis from the dermis by dispase treatment overnight, lysis of the epidermal layer and subsequently by following the same procedure as for WBC.

The above-mentioned procedures for the isolation of DNA from WBC and skin biopsies are very laborious and time-consuming. We attempted several modifications to simplify and to speed up this procedure, using various commercially available kits. The most important advantages of these procedures are the small amount of sample required (only 300 µl of blood or 10-20 mm^2 of epidermis) and the decrease in labour and time needed in comparison to the originally applied phenolic extraction method. Results obtained with a number of these kits are presented in Figure 1.

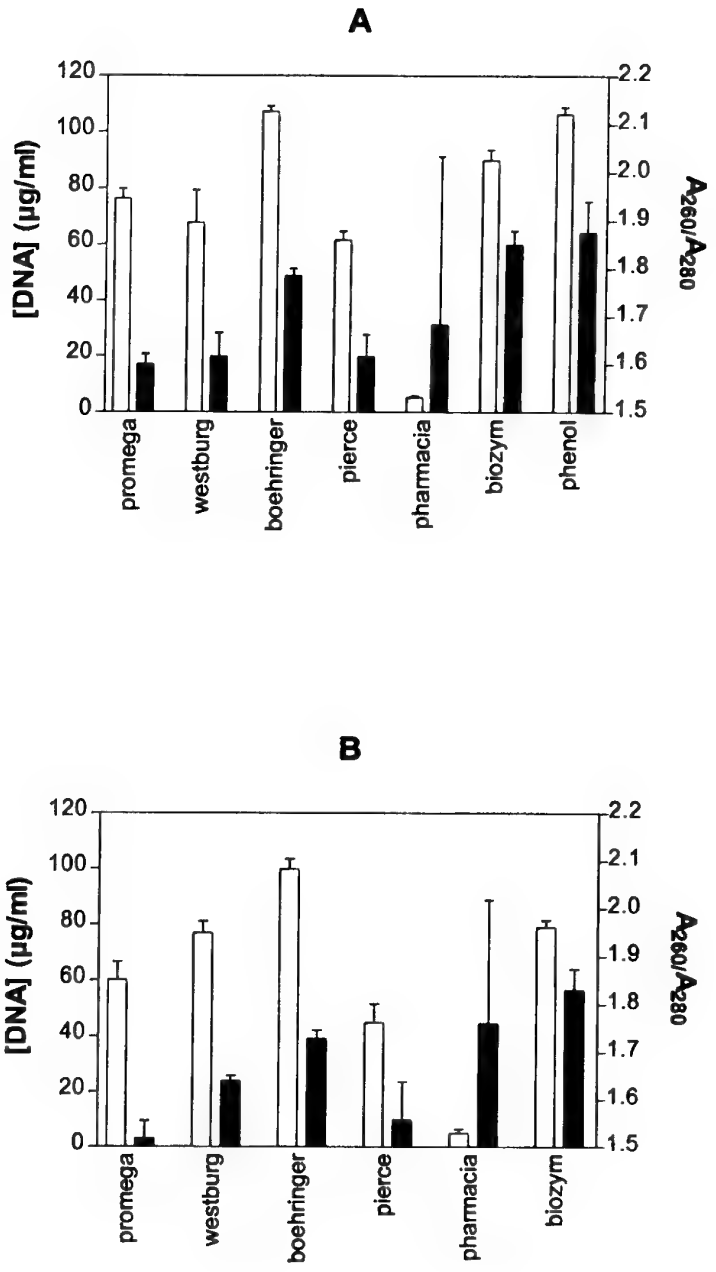

Figure 1. Yield (µg/ml final solution, open bars) and purity (A_{260}/A_{280}, filled bars) of DNA after isolation from human blood (300 µl) using various commercial kits (see Subsection III.1.3). DNA was dissolved in 100 µl TE buffer after the final DNA precipitation step. Panel A: fresh blood, panel B: frozen blood. All isolation procedures were carried out in quadruplicate. The data represent the mean with standard deviation. For comparison, the yield and purity of DNA are shown after isolation from fresh human blood (1 ml) using the original phenol extraction method. In the latter case the DNA was dissolved in 300 µl TE buffer after the final DNA precipitation step.

The amount of DNA obtained from the blood samples was 7-10 μg and the A_{260}/A_{280} ratio[3] ranged between 1.7 and 1.9, both for fresh blood and frozen blood. Comparable results were obtained by applying the very laborious phenol extraction method to fresh blood. This method applied to frozen blood resulted in very slowly dissolving DNA pellets after the final precipitation step and extremely low yields (data not shown). The lysis of the WBC from frozen blood took some more time than those from fresh blood. The best results were obtained with the DNA isolation kits of Biozym and Boehringer yielding reasonable amounts of DNA and an acceptable purity.

A drawback of these procedures is the rather long time that is still needed to lyse the cells and to dissolve the DNA precipitate. Currently, it is studied whether less tight pelleting of the WBC after erythrocyte lysis without substantial loss of cells will speed up the lysis of the WBC. In addition, the ease to dissolve the DNA precipitate might be dependent on the degree of drying the DNA pellet; overdrying will make the DNA pellet difficult to dissolve. It will be studied to which extent drying can be omitted without disturbing the further procedure in the immunoslotblot assay.

Both the DNA isolation kits of Biozym and Boehringer were applied to blood exposed to sulfur mustard (in a range of 0.1 to 10 μM). The results indicated (data not shown) that after sulfur mustard exposure of human blood DNA can be isolated in approximately the same amounts as those obtained from unexposed blood and at a similar A_{260}/A_{280} ratio (1.7 to 1.9). In general, isolation of DNA from frozen blood appeared to be more difficult than from fresh blood due to the impaired lysis of the WBC.

The adduct levels in the DNA preparations obtained with the DNA isolation kits of Biozym and Boehringer, as detected with the immunoslotblot assay, were at least the same or even somewhat higher than in the DNA preparations isolated in the conventional way (data not shown).

With the commercial kit of Biozym, DNA was also isolated from human skin biopsies. To this end, the epidermis was first separated from the dermis by an overnight treatment with the enzyme dispase and then treated with the Cell Lysis Buffer following the same procedure as for WBC. A skin biopsy of 10-20 mm^2 appeared to be sufficient to yield 10-20 μg of DNA.

IV.1.3 Simplification and improvement of immunoslotblot procedure for N7-HETE-Gua

In the immunoslotblot assay (ISB) the single-stranded DNA containing N7-HETE-Gua was first slotblotted onto a nitrocellulose filter. After blotting, the slots were rinsed with PBS. Originally, the next step was baking at 80 °C to immobilize the DNA. In the modified protocol, the filters were dried on air and the DNA was immobilized by UV crosslinking. This modification resulted in about 10-fold enhancement of the chemiluminescence signal. The amount of DNA blotted appeared to be critical. Approximately, a 2-fold increase in the amount of DNA resulted in a 4-fold increase of the chemiluminescent signal (Figure 2). For that reason we decided to blot standardly 1 μg DNA/blot instead of various amounts of DNA as applied in previous experiments and to reserve 10 positions on the 96-blots filter for calibration samples of DNA with adduct levels in the range of 0-10 N7-HETE-Gua/10^7 nucleotides.

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[3] A higher ratio indicates a higher purity

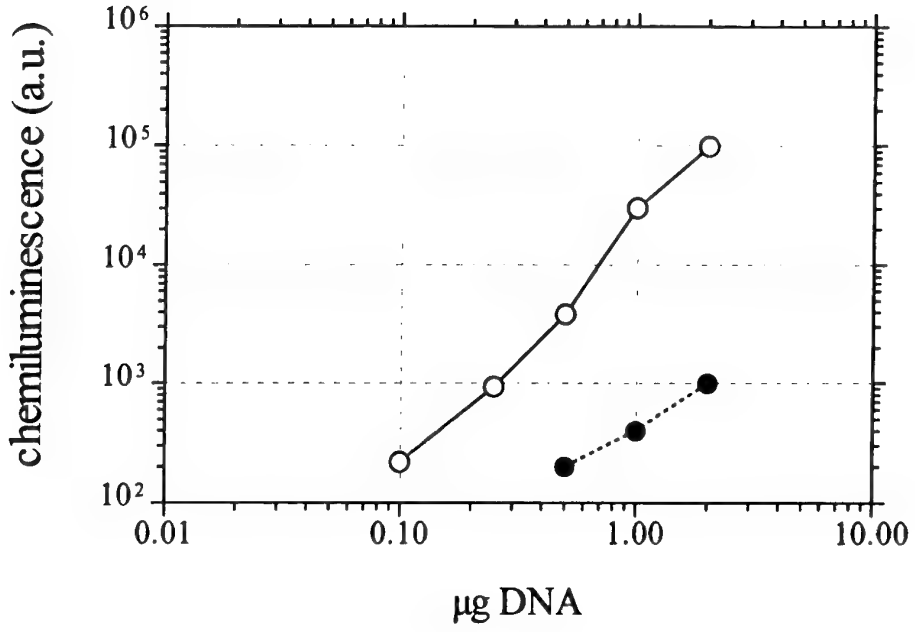

Figure 2. Immunoslotblot assay for the detection of N7-HETE-Gua in double stranded calf thymus DNA (ds-ct-DNA) exposed to 0 (●) or 2.5 (O) nM sulfur mustard (30 min, 37°C): dependence of chemiluminescence on the amount of DNA used in the assay. The data points represent the averages of the chemiluminescence (in arbitrary units) of two samples. Ds-ct-DNA (50 µg/ml) was made single-stranded by heating for 15 min at 52 °C in TE buffer containing 4% formamide and 0.1% formaldehyde.

With respect to the substrate solutions of Boehringer, it appeared to be important to mix the solutions A and B and to equilibrate for 1 h at 25 °C before addition to the filter (the manufacturer did not provide a clear instruction about that aspect). Since we now use a luminometer instead of cassettes with photographic film, the handling of the filters has also been modified. In this modified procedure, the filters were incubated for 1 min in substrate and then placed in a plastic bag. Excess of liquid was pressed out and the filters were placed in the luminometer to measure chemiluminescence.

A time-consuming step in the procedure is the overnight incubation with the 1st antibody at 4 °C. In an alternative assay, incubation with the 1st antibody was carried out for 2 h at 37 °C. This resulted in a lower sensitivity. The extent of impairment is still under study.

IV.1.4 Simplification of signal detection of the ISB procedure for N7-HETE-Gua

Originally, the chemiluminescence signal was measured by exposure of a photographic film to the blotted filters for 5-120 s. The signal was quantified by scanning of the developed film with a densitometer. Two drawbacks of this procedure were the non-linear blackening characteristics of the photographic film and the rather long time required to quantify the blackening. The purchase of a 1450 MicroBeta Trilux luminescence counter having six simultaneously operating detectors appeared to be a significant improvement in both aspects. The response to the chemiluminescence signal over 1 s is proportional over at least 4 decades. All 96 blots are quantified within 1 min after start of the scanning. Scanning can be started immediately after

placing the filters in the plastic bags and transferring these to the cassette of the device. The chemiluminescence signal is constant over a period of at least 30 min. Initial problems with the exact positioning of the filter in the cassette could be solved by adding markers on the filter. An example of a dose-effect curve is presented in Figure 3. These results clearly demonstrates the linear relationship between the chemiluminescence measured and the sulfur mustard concentration to which DNA was exposed, which could not be achieved with a photographic film. Moreover, an enhancement of chemiluminescence could be observed for DNA treated with 2.5 nM sulfur mustard relative to untreated DNA, whereas the lower detection limit in previous experiments was at about 10 nM sulfur mustard.

The lower detection limit in the modified assay still showed some variation which may be in part due to day-to-day variations in the state of the chemiluminescence blotting detection system used. Nevertheless, it could be derived that the lower detection limit was in a range of 8-40 amol N7-HETE-Gua/blot with 1 μg DNA. This corresponds to an adduct level of 3-13 N7-HETE-Gua/10^9 nucleotides.

Some attention was required in the case of assays performed with samples containing a large amount of N7-HETE-Gua. This may cause saturation of the detector system and may lead to crosstalk of chemiluminescence to the neighbouring blots (about 0.2%). The first problem can be solved by application of samples diluted with DNA not exposed to sulfur mustard or by application of a grey filter. The effect of crosstalk can be avoided by not using the neighbouring blots or also by diluting samples with unexposed DNA. According to the manufacturer the use of a red filter should also have the advantage of decreasing crosstalk.

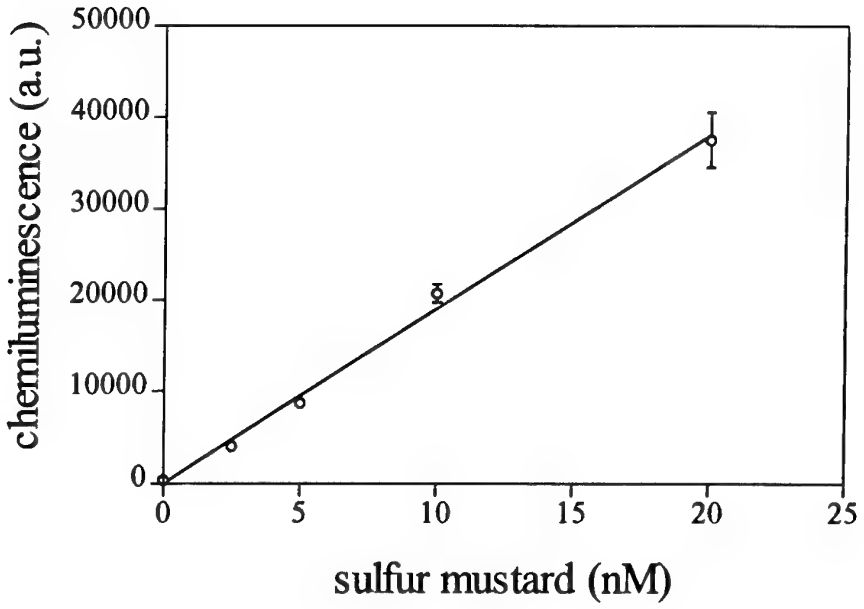

Figure 3. Immunoslotblot assay of N7-HETE-Gua in ds-ct-DNA exposed to various concentrations of sulfur mustard for 30 min at 37 °C. The data points represent the average of the chemiluminescence (in arbitrary units) of two samples. The error bars represent the range between those. Ds-ct-DNA was made single-stranded by heating for 15 min at 52 °C in 10 mM Tris buffer containing 1 mM EDTA, 4% formamide and 0.1% formaldehyde.

IV.1.5 Effect of conditions for sulfur mustard treatment of DNA and blood on the induction of N7-HETE-Gua

At the start of this study the lower detection limit of the immunoslotblot assay for exposure of human blood was 70 nM sulfur mustard. This corresponds to an adduct level of 300 N7-HETE-Gua/10^9 nucleotides. Since the lower detection limit for ds-ct DNA treated by sulfur mustard was substantially improved by employing the modified ISB procedure (Subsection IV.1.4), we intended to determine concentration-effect curves for *in vitro* exposure of human blood to sulfur mustard including a lower concentration range, using the same procedure. In these experiments blood was mixed with a diluted solution of sulfur mustard at room temperature and the reaction was terminated at 1 h after administration.

The data obtained (Figure 4) indicate that the levels of N7-HETE-Gua determined in blood exposed to sulfur mustard were lower than those observed previously (7) over the whole concentration range. Even at 100 nM sulfur mustard, N7-HETE-Gua levels were only slightly increased in comparison to those in untreated blood, in spite of the increased sensitivity of the assay. In addition, the ratio between adduct levels found in blood treated with 10 µM and 1 µM sulfur mustard appeared to be more than the expected factor of 10 (Figure 4). Therefore, some modifications were carried out to the treatment conditions of blood with sulfur mustard: (i) blood was added to the sulfur mustard dilution and *vice versa*, (ii) after mixing at room temperature the incubation mixture was left at room temperature for 1 h and placed in an incubator (37 °C), and (iii) blood collected in heparin and in EDTA was used. The results summarized in Figure 4

indicate that addition of blood to sulfur mustard instead of sulfur mustard to blood did not eliminate the unexpected concentration-effect observed after treatment with sulfur mustard at low concentrations. Incubation at 37 °C after mixing seemed to be an improvement. The use of heparin instead of EDTA seemed to have also a positive effect on the linearity of the concentration-effect relation. However, drawbacks of the use of heparin instead of EDTA appeared to be difficulties with solving the DNA pellet after isolation of the DNA and the decreased purity reflected in a lower A_{260}/A_{280} ratio. When WBC cells (purified by lysis of the erythrocytes) were treated with sulfur mustard, the linearity was also better than with blood. These data suggest that the reaction temperature influences the linearity of the concentration-effect curve of sulfur mustard exposure of human blood.

Because incubation at 37 °C seemed to have a positive effect on the linearity of the concentration-effect relation, experiments will be repeated such that blood samples are pre-warmed to 37 °C before mixing with the sulfur mustard dilutions, followed by incubation at 37 °C. Possibly, this will result also in a relatively higher adduct level after treatment in the concentration range of 0 to 100 nM sulfur mustard.

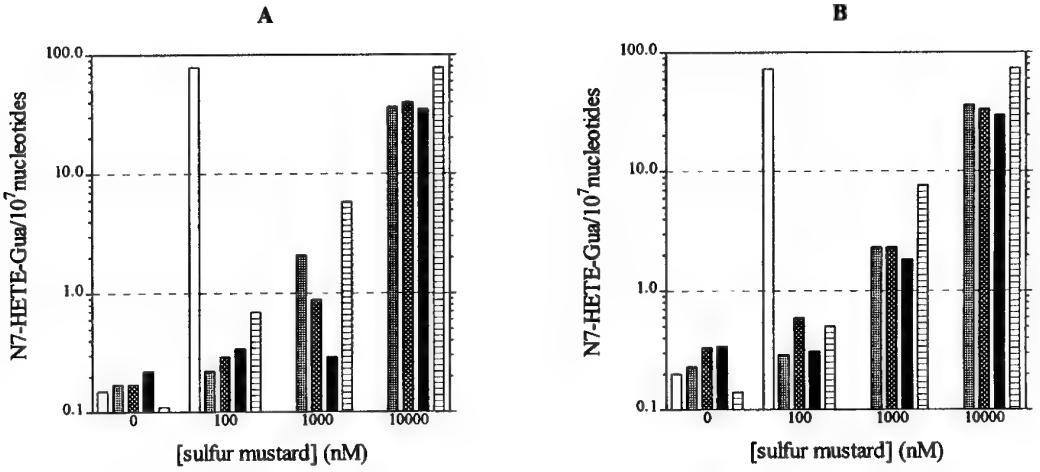

Figure 4. Immunoslotblot assay of N7-HETE-Gua in DNA of WBC of human blood collected in EDTA (panel A) and in heparin (panel B) that was exposed to sulfur mustard at various conditions. Human blood was mixed with an appropriate sulfur mustard dilution at room temperature and the incubation mixture was either immediately placed in an incubator at 37 °C (▒) or left at room temperature (▒ : blood added to sulfur mustard solution; ▒ : sulfur mustard solution added to blood). For comparison, double-stranded calf thymus DNA (open bars) and WBC (bars with horizontal lines) have been exposed to sulfur mustard at room temperature. The data represent the average of the adduct level derived from the chemiluminescence of two samples in relation to that of calibration DNA samples. The estimated error ranged from about 0.1 N7-HETE-Gua/10^7 nucleotides for the samples not exposed to sulfur mustard to about 5 N7-HETE-Gua/10^7 nucleotides for the highest concentrations presented.

IV.1.6 Day-to-day variability of ISB for N7-HETE-Gua in DNA in a single blood sample

As described already in Subsection IV.1.4, the lower detection limit for the detection of N7-HETE-Gua in DNA varied within a certain range due to day-to-day variations in the blotting detection system. In addition, the day-to-day variability in the level of N7-HETE-Gua in DNA measured in the same DNA sample isolated from sulfur mustard treated blood was sometimes more than 20%. Some improvement seemed to be achieved in the DNA isolation and denaturation procedure by making the DNA solution more homogeneous through repeated freezing-thawing both after solving the DNA-precipitate and after the denaturation procedure. Whether this is the only solution of the problem is still under study.

IV.2 Development of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin as a Standard Operating Procedure

IV.2.1 Introduction

A number of experiments were carried out to optimize the procedure for a modified Edman degradation with respect to sensitivity and simplicity, as a part of the development of a standard operating procedure for the determination of sulfur mustard adducts to the N-terminal valine in hemoglobin. ^{14}C-labeled sulfur mustard was advantageously used in some of these experiments as well as in experiments described in other sections of this chapter. Results of the synthesis of the compound are given in this section

IV.2.2 Synthesis of [^{14}C]sulfur mustard

In the final report of the previous grant (7) we reported that the synthesis of [^{35}S]sulfur mustard was troublesome. The yield and the purity of the obtained product varied considerably for various synthetic runs. The bottle-neck of the synthesis was probably the purity of the [^{35}S]hydrogen sulfide. Therefore, we focussed our attention on the synthesis of [^{14}C]sulfur mustard, which has the additional advantage of the long half life of the ^{14}C-isotope.

As starting material we chose commercially available [^{14}C]bromoacetic acid. Reduction with borane tetrahydrofuran complex solution in THF afforded [^{14}C]bromoethanol, which was used without further purification (Figure 5). Reaction of the latter with Na_2S afforded [^{14}C]thiodiglycol in moderate yield, which could be isolated by silica gel column chromatography. The major disadvantage of this procedure was the concomitant formation of the ^{14}C-labeled disulfide of mercaptoethanol, which could not easily be removed by silica gel column chromatography since this compound has a similar retention as thiodiglycol. This problem could be circumvented by reaction of [^{14}C]bromoethanol with 2-mercaptoethanol under the agency of sodium ethoxide; the disulfide which was formed in this case was not radioactive. [^{14}C]Thiodiglycol was obtained in 54% yield. Finally, conversion of [^{14}C]thiodiglycol into [^{14}C]sulfur mustard was effected by reaction with thionyl chloride. The crude sulfur mustard obtained was contaminated with a radioactive compound with a longer retention time with GC analysis. In order to isolate [^{14}C]sulfur mustard, the crude sample (obtained from 0.27 mmol [^{14}C]thiodiglycol) was diluted with cold sulfur mustard (0.2 mmol) and then distilled. Two batches of [^{14}C]sulfur mustard were obtained with a radiochemical purtity > 99% and a specific activity of 15 mCi/mmol.

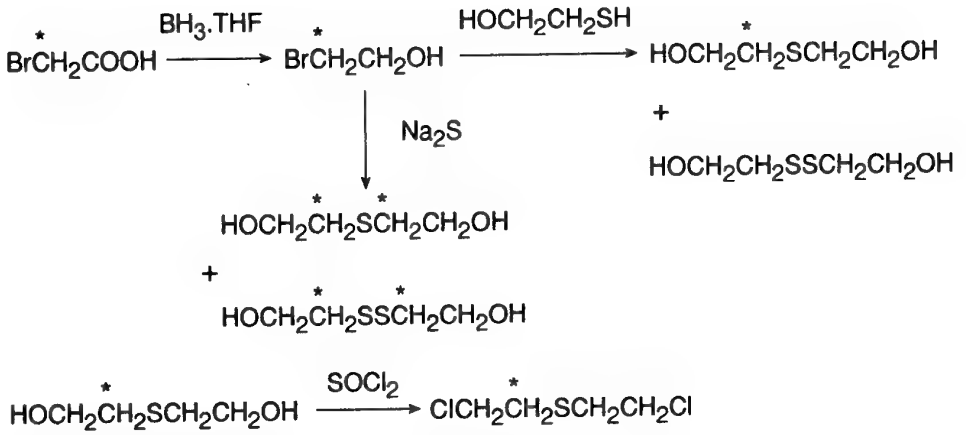

Figure 5. Synthesis of [^{14}C]thiodiglycol containing either one or two radioactive labels (C*) and the subsequent formation of [^{14}C]sulfur mustard containing one radioactive label.

IV.2.3 Simplification of the modified Edman procedure

The first step in the analysis of adducted N-terminal valine in hemoglobin is the isolation of globin. In an attempt to shorten the procedure by leaving out this isolation step, hemolysates of human blood that was exposed with 10 µM of sulfur mustard were treated with the modified Edman reagent. However, sulfur mustard adducts could not be detected by GC-NCI/MS analysis of the samples obtained after further processing of the treated hemolysates in the usual way.

Next some modifications were introduced into the modified Edman procedure itself in order to simplify and shorten the procedure. The degradation step was performed by reaction for 2 h at 60 °C instead of incubation overnight at room temperature followed by reaction for 2 h at 45 ° C. Furthermore, the reaction mixture was worked up by extraction with toluene only, leaving out the first extraction step with ether. Both the original and the simplified procedure were used for processing of globin which had been isolated from blood exposed to ^{14}C-labelled sulfur mustard (1 mM). We could not detect a difference in radioactive thiohydantoin upon HPLC analysis with radiometric detection. In the second year of the grant period, the detection limits of the two procedures for exposure of human blood to sulfur mustard will be compared.

IV.2.4 Enhancement of the sensitivity of the modified Edman procedure

Two approaches were followed in order to lower the detection limit for the modified Edman procedure. In the first approach, GC-NCI/MS of the final sample was performed using a TCT injection technique. As a preliminary step for such a TCT injection, the sample was applied onto Tenax absorption material. After venting most of the solvent by a stream of helium, the analytes are thermally desorbed and transferred into a cold trap. The analytes are injected onto the analytical column by flash heating of the cold trap. Much larger sample volumes (e.g., 50-100 µl) can be used with this injection technique than with a normal injection (sample volume 1-3 µl). The detection limit of synthesized thiohydantoin of N-HETE-valine derivatized with a heptafluorobutyryl group was determined to be 100 fg when a sample volume of 50 µl was

applied. By using this injection technique (sample volume 50 µl), the detection limit of the modified Edman procedure for exposure of human blood to sulfur mustard was lowered from 100 to 30 nM of the agent.

In the second approach, attempts were made to lower the detection limit by purification of the crude thiohydantoin obtained after the modified Edman degradation, by means of solid phase extraction procedures. Firstly, Florisil cartridges containing straight phase silica gel were used, since it is advantageous to obtain the samples as solutions in an anhydrous, apolar solvent for introduction of the heptafluorobutyryl group. Globin samples were used which had been isolated from blood exposed to ^{14}C-labeled sulfur mustard (1 mM). The purification step was followed by HPLC with radiometric and UV detection. Only a minor loss (< 2%) of the thiohydantoin was observed. The UV pattern demonstrated that a significant purification could be obtained by inserting this relatively simple purification step (see Figure 6). A purification

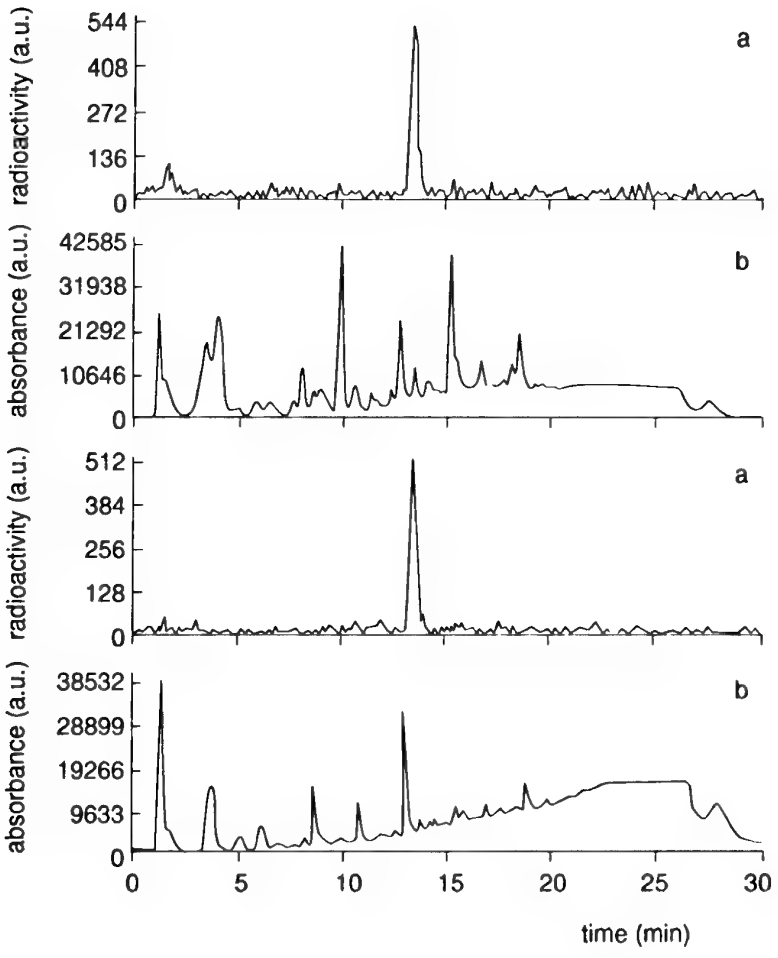

Figure 6. HPLC chromatogram (PEP-RPC 5/5 column) of thiohydantoin obtained after modified Edman degradation of globin isolated from human blood that was exposed to [^{14}C]sulfur mustard (1 mM), before (2 upper panels) and after solid phase extraction with a Florisil cartridge (two lower panels). A, detection of radioactivity; B, UV detection (254 nm). Eluent: 0.1% TFA in water with a linear gradient to 0.1% TFA in acetonitril/water (80/20, v/v) in 20 min.

with Sep-Pak C18 gave a similar outcome, although the loss of thiohydantoin was larger (13%). Combining the two purification steps afforded an even more purified sample. It is expected that larger batches of globin can be processed by applying this purification step, without resulting in a higher level of impurities in the final sample. This will allow us to detect lower exposure levels. Experiments along these lines will be performed in the second year of the grant period.

IV.3 Detection of hemoglobin adducts

IV.3.1 Introduction

The following steps were taken in our general approach to the development of an immunochemical assay for the detection of sulfur mustard adducts with hemoglobin. It has been attempted to further improve the sensitivity of the immunochemical assay by using the antibodies obtained in the previous agreement (7) which were raised against S-HETE-cys_{93} of the β-chain of human hemoglobin. In addition, a synthon derived from adducted N1/N3-histidine was synthesized which was found to be the most abundant adduct formed in hemoglobin after exposure of human blood to sulfur mustard (7). Three alkylated peptides representing partial sequences of hemoglobin and containing the adducted histidine were synthesized and served as haptens for raising antibodies. Studies on the development of an immunochemical assay based on these antibodies will be performed in the second and third year of the agreement.

IV.3.2 Characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin

Several clones of which the antibodies recognized alkylated hemoglobin were obtained from mice immunized with an alkylated peptide, i.e., N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin (7). One of these, 3H6, was further characterized. These antibodies recognize hemoglobin in a dose-dependent way. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA (7). However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have now attempted to apply these antibodies to an immunoslotblot assay for alkylated hemoglobin, but so far without lowering the detection limit.

IV.3.3 Synthesis of peptide haptens containing a histidine-sulfur mustard adduct

During the previous grant it was found that N1/N3-HETE-histidine is the most abundant adduct formed in hemoglobin after exposure of human blood to sulfur mustard. Three specific histidine residues were identified that are alkylated by sulfur mustard, i.e., α-his_{20}, β-his_{77} and β-his_{97}. We here describe the synthesis of peptide haptens, derived from human hemoglobin, containing these alkylated residues. The required building block was synthesized starting from Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester, the synthesis of which was described in the final report of grant DAMD17-92-V-2005 (7). The Boc group was selectively removed under the agency of dry HCl (1 M) in ethyl acetate. Subsequently, the ester function was saponified in methanol/water containing 0.2 M NaOH and finally the Fmoc group was introduced according to a published procedure, affording Nα-Fmoc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine in 70% yield.

The following peptides were synthesized:

1. A-F-S-D-G-L-A-H(N1/N3-HETE)-L-D-N-L-K, which represents the amino acid residues 70-82 of human β-globin

2. G-K-V-G-A-H(N1/N3-HETE)-A-G-E-Y-G-A-K, which represents the amino aicd residues 15-26 (+ lysine) of human α-globin
3. L-H(N1/N3-HETE)-V-D-P-E-N-F-R-L-L-G-N-V-K, which represents the a.a residues 96-109 (+ lysine) of human β-globin.

FPLC analysis showed the presence of one main product in each case. Electrospray MS analysis showed the presence of the expected mass and the sequence of the peptides was firmly established by means of tandem MS analysis. The corresponding native sequences were also synthesized and will be used as reference compounds in immunochemical experiments with antibodies raised against the three N1/N3-HETE-histidine-containing peptides.

IV.3.4 Antibodies against peptide haptens containing a histidine-sulfur mustard adduct

We immunized mice with the three different peptide haptens containing a histidine-sulfur mustard adduct, described in Subsection IV.3.3. Subsequently, these mice were used for fusion experiments with the following preliminary results. Clones were selected on their capacity to produce antibodies with specificity for hemoglobin treated with 50 μM sulfur mustard. With hapten 1 two clones were obtained. With hapten 2 one clone was selected and with hapten 3 five clones after subcloning of one clone. Antibodies of these clones (except the one obtained with hapten 1) were tested for specificity on hemoglobin treated with 50 μM sulfur mustard and on keratin treated with 50 or 500 μM sulfur mustard (Table 1). These antibodies show specificity not only for alkylated hemoglobin but also for alkylated keratin. In one case, clone 190-2H12, the specificity for alkylated keratin seemed to be even higher than for alkylated hemoglobin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Table 1. Antibody specificities of clones obtained from a fusion after immunization with two peptide haptens containing a histidine-sulfur mustard adduct[a]. Supernatants of cultures were assayed in a direct ELISA on keratin treated with 50 or 500 μM sulfur mustard or on hemoglobin treated with 50 μM sulfur mustard. +: fluorescence significantly higher than that obtained on the non-alkylated protein.

Clone	Hemoglobin treated with 50 μM sulfur mustard	Keratin treated with 50 μM sulfur mustard	keratin treated with 500 μM sulfur mustard
190-4A3	+	+/-	++
186-1A4	++	+	+
183-5B7	++	+	+
183-3D5	+	-	+
190-4F5	+/-	-	+
190-2H12	-	+/-	+
190-5E7	+	-	-
3H6 (control)	-	+/-	-

[a] Haptens 1 and 3 as described in Subsection IV.3.3.

In this experiment, the control clone, 3H6, appeared to be negative which suggests that the test system, particularly the coating of the microtiter plates, was still not optimal. Nevertheless, several other clones were positive, suggesting that these clones produced antibodies which were more specific than those of 3H6. During selection of the clones the direct ELISA appeared to be not sufficiently reproducible. Presently, efforts are being made to improve it.

IV.4 Detection of albumin adducts

IV.4.1 Introduction

In order to develop an immunochemical assay for the detection of sulfur mustard adducts with albumin, i.e., the most abundant protein in plasma, the following steps were taken in our general approach: quantitation of the binding of the agent to the protein by using [^{14}C]sulfur mustard and analysis of tryptic digests of albumin that was exposed to sulfur mustard, for identification of alkylation sites in the protein. One of the alkylated peptides, i.e., the fragment T5 containing an alkylated cysteine, could sensitively be detected in the tryptic digest with LC-MS-MS analysis. Therefore, this alkylated peptide has been synthesized and will serve as a hapten for raising antibodies. In the second year of the grant period, the feasibility of analysis of this alkylated peptide in tryptic digest of exposed albumin will be further investigated for retrospective detection of exposure to sulfur mustard.

IV.4.2 Quantitation of binding

Two methods for isolation of albumin from whole blood were examined. The first method (20,21), employing ammonium sulfate precipitation, was quite laborious. The second method (17), based on successive precipitations of fibrinogen, globulins, and albumin, was more simple. The purity of the albumin isolated was assessed by SDS-PAGE; both methods gave albumin of high purity (95%).

For quantitation of sulfur mustard binding to the protein, blood was exposed to 1300, 130, 13 and 1.3 µM of ^{14}C-labeled agent (2 h at 37 °C). After isolation of albumin, the protein (2 mg) was dissolved in a solution of 1 M urea in 0.9% NaCl and radioactivity was determined with liquid scintillation counting. As the specific activity of [^{14}C]sulfur mustard (15 mCi/mmol) and the molecular weight of albumin (66.5 kDa) are known, the amount of radioactive material covalently bound per mol of protein could be calculated from the results. A survey of the results is given in Table 2. The results are comparable to binding data obtained for hemoglobin

Table 2. Binding of [^{14}C]sulfur mustard to human serum albumin upon treatment of human blood with various concentrations of the agent

Concentration [^{14}C]sulfur mustard (µM)	[^{14}C]sulfur mustard bound to albumin per ml blood[a] (nmol)	µmol [^{14}C]sulfur mustard bound per 1000 µmol of albumin
1.3	0.27 (21)	0.43
13	2.6 (20)	4.1
130	26 (20)	41
1300	230 (18)	370

[a] Data within parentheses denote the percentages of total radioactivity added to blood that was bound to albumin

as determined previously (7). The binding to both proteins is linear with the sulfur mustard concentration, whereas the percentage of sulfur mustard that is bound to the proteins is of the same order, i.e., 20% and 25% for albumin and hemoglobin, respectively.

IV.4.3 Mass spectrometric identification of alkylation sites for sulfur mustard in albumin

In the final report of our previous grant (7) we described the use of advanced mass spectrometric techniques for analysis of hemoglobin alkylated by sulfur mustard. We identified several sites of alkylation by sulfur mustard within the tertiary structure of hemoglobin, using tandem mass spectrometry combined with micro-LC. In this part we investigated the feasibility of tandem mass spectrometry to identify alkylation sites for sulfur mustard in albumin.

In order to obtain efficient digestion of albumin, the disulfide bridges present in the protein were reduced with dithiothreitol and the resulting free cysteine residues were alkylated with iodoacetic acid. Subsequently, the protein was digested with trypsin. HPLC analysis of tryptic digests of albumin isolated from human blood that was treated with sulfur mustard gave reproducible chromatograms. When albumin was used which was isolated from blood that had been treated with [^{14}C]sulfur mustard, a large number of radioactive peaks were observed, demonstrating efficient alkylation of albumin by sulfur mustard. The large peak in the early region of the chromatogram probably represents [^{14}C]thiodiglycol (cleavage of ester adducts) and small alkylated peptides. Fortunately, one peak in the late-eluting region of the chromatogram, containing 4-5% of total radioactivity, was fully separated from other peptide material (Figure 7). With higher exposure levels an additional peak in the UV region could be observed which coincided with the radioactive peak.

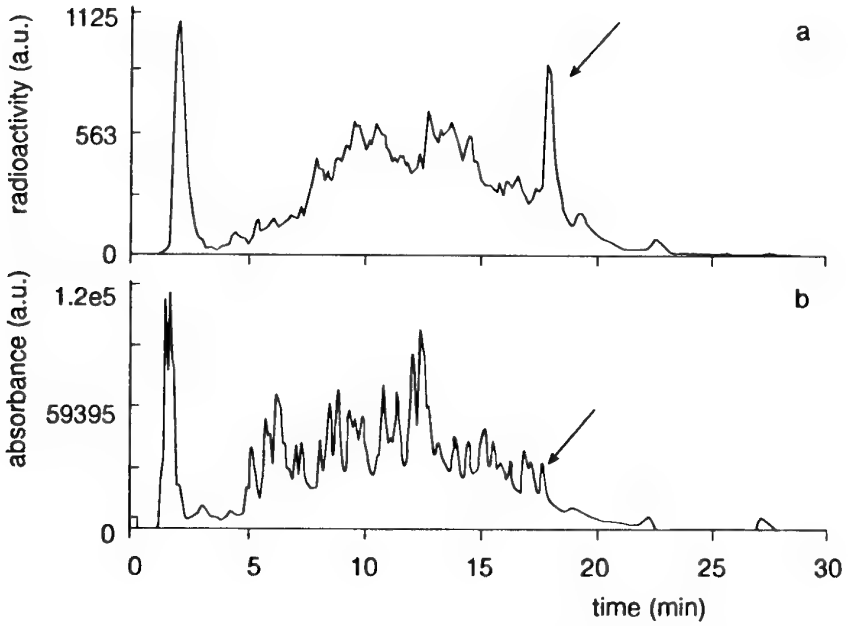

Figure 7. HPLC chromatogram (PEP-RPC 5/5 column) of a tryptic digest of albumin isolated from human blood that was treated with [^{14}C]sulfur mustard (10 mM). A, detection of radioactivity; B, UV detection (254 nm). Eluent: 0.1% TFA in water with a linear gradient to 0.1% TFA in acetonitril/water (48/52, v/v) in 20 min. The arrow indicates the peak for the alkylated T5 fragment.

Since this peptide represented a relatively high percentage of the total radioactivity bound to albumin and was fully separated from other peptides, our attention was focussed on the identification of this compound. Mass spectrometric analysis of a tryptic digest of albumin from blood exposed to 10 mM sulfur mustard (Figure 8) showed the presence of a compound with *m/z* 1269.3, which corresponds with $[MH_2^{2+}]$ of the alkylated T5 fragment, i.e., HETE-(A-L-V-L-I-A-F-A-Q-Y-L-Q-Q-C-P-F-E-D-H-V-K) ($MW_{monoisotopic}$ 2536 Da, $MW_{average}$ 2538 Da). The T5 fragment contains the only free cysteine residue of the protein (at position 34) which is believed to be highly reactive towards electrophiles (17). Tandem MS experiments showed that alkylation had indeed occurred at cysteine-34, which is clearly demonstrated from the *m/z* values of the fragments Y''_7 and Y''_8 corresponding to values for a nonalkylated and an alkylated fragment, respectively (Figure 9). Moreover, the radioactive peak of the peptide in the tryptic digest coeluted with synthetic T5 alkylated with sulfur mustard at the cysteine, which was readily available by solid phase synthesis. Previously (7), peptides containing a cysteine-sulfur mustard adduct were synthesized employing a building block in which the HETE group was protected with a *tert*-butyl group. We now found that the hydroxyl function can be left unprotected (at least for this particular sequence), i.e., employing N-Fmoc-S-HETE-cysteine as a building block for solid phase peptide synthesis. The resulting crude product consisted mainly of the desired S-alkylated T5 and was used without further purification.

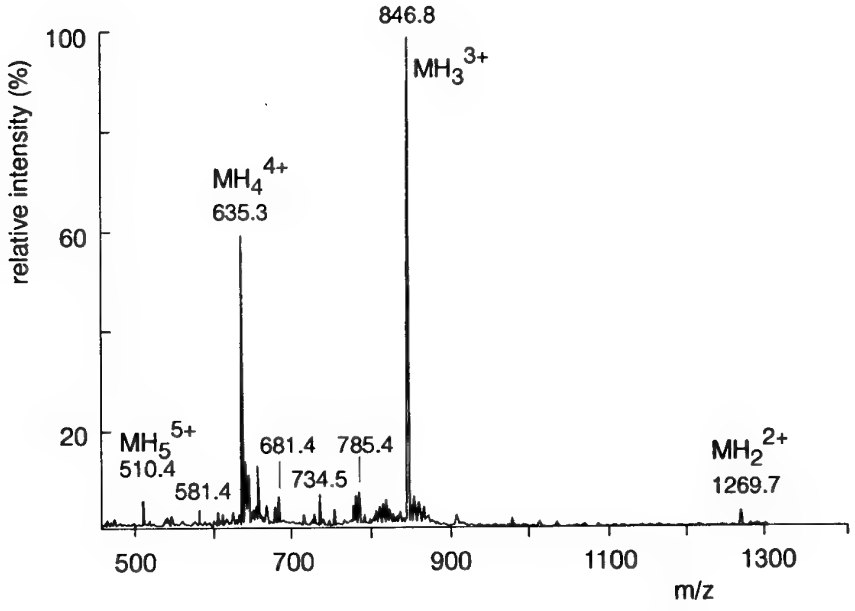

Figure 8. Mass spectrum upon electrospray LC-MS analysis of T5 peptide in a tryptic digest of albumin isolated from human blood that was exposed to 10 mM sulfur mustard.

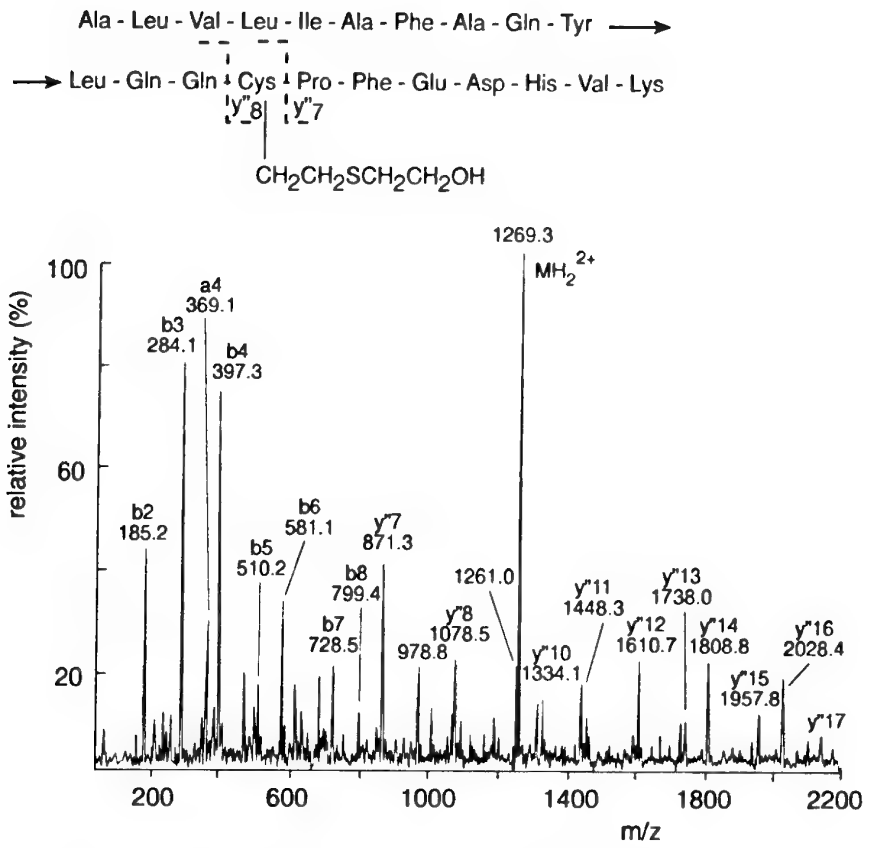

Figure 9. Tandem MS spectrum for molecular ion MH_2^{2+} (*m/z* 1269, see also Figure 8) of alkylated T5 peptide in a tryptic digest of albumin isolated from human blood that was exposed to 10 mM sulfur mustard.

The synthetic alkylated peptide will serve as a hapten for raising antibodies against albumin that has been exposed to sulfur mustard. It also seems worthwhile to investigate whether LC tandem MS analysis of this peptide in a tryptic digest of albumin is suitable for retrospective detection of exposure to sulfur mustard. As a first step, the detection limit of LC tandem MS analysis was determined for the synthetic compound. The detection limit of selective ion recording (SIR) for *m/z* 1269.5 (MH_2^{2+}) was 10 pg of the alkylated peptide. In the MRM mode the following transitions were recorded:

m/z 846.3 (MH_3^{3+}) → *m/z* 1071.0, 1014.5, and 978.5
m/z 846.3 (MH_3^{3+}) → *m/z* 185.0, 284.2, and 397.3
m/z 1269.5 (MH_2^{2+}) → *m/z* 185.0, 284.2, and 397.3

The most sensitive analysis was obtained from combined recording of the first three transitions, i.e., *m/z* 846.3 → *m/z* 1071.0, 1014.5 and 978.5, allowing a detection of ≥ 15 pg of the alkylated peptide.

For analysis of trypsinized albumin samples, MRM with high resolution was the method of choice since SIR was not specific enough. The detection limit for the adduct was now increased to 45 pg (from standard addition, S/R 3:1). The detection limit for in vitro exposure of human blood was determined to be 1 μM (Figure 10). Unfortunately, serious problems were encountered with blank samples, since small signals (just above the detection limit for alkylated T5) were observed at the same retention time as the alkylated T5 fragment. Since

impurities in the iodoacetic acid might be responsible for the interferences in the blank, the reduction and alkylation of the isolated albumin were omitted. Unfortunately, the detection limit for the alkylated T5 fragment deteriorated severely because of peak broadening. Some of the analyses were performed in the U.K. using a Q-TOF-MS. This technique allows to acquire a full scan MS-MS spectrum of the peptide analyte at the same sensitivity as provided by an electrospray MS-MS analysis under MRM conditions. In the near future, a similar apparatus will be available in TNO Prins Maurits Laboratory. Mass spectrometric analysis of this alkylated peptide in tryptic digest of exposed albumin will be further investigated in the second year of the grant period, including analyses performed with the Q-TOF-MS.

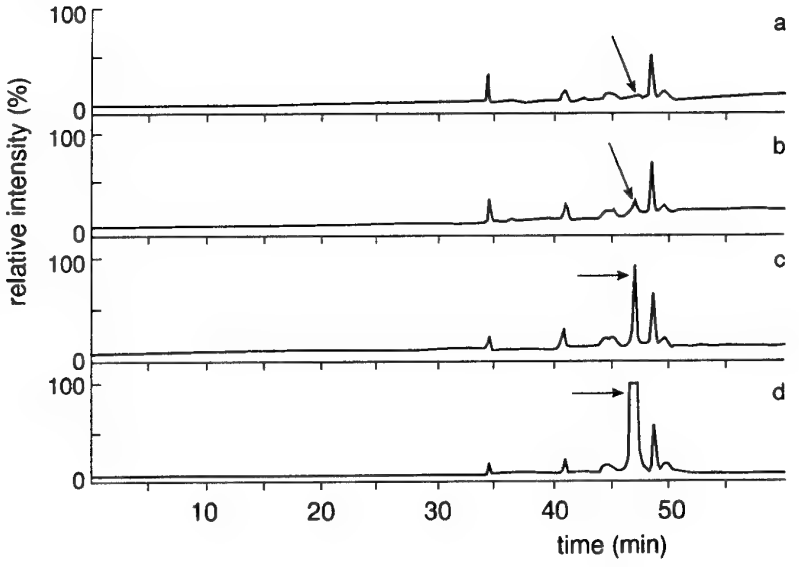

Figure 10. LC tandem MS analysis of T5 peptide (arrow) in a tryptic digest of albumin, using the multiple reaction monitoring scanning mode for the transition *m/z* 846 (MH_3^{3+}) → 1071. Albumin was isolated from non-exposed human blood (A) and from human blood that was exposed to 1 μM (B), 10 μM (C), and 100 μM (D) of sulfur mustard.

IV.5 Detection of keratin adducts

IV.5.1 Introduction

First steps similar to those for albumin adducts (see Section IV.4) were taken in order to develop an immunochemical assay for the detection of sulfur mustard adducts with keratin, i.e., the most abundant protein present in human epidermis and stratum corneum. Binding of the agent to the protein was quantitated by using [^{14}C]sulfur mustard and keratin that had been exposed to sulfur mustard was hydrolyzed enzymatically. Although the latter approach did not give satisfactory results, results obtained in the first series of experiments provided sufficient information both to design promising haptens for raising antibodies against keratin exposed to sulfur mustard and to develop a mass spectrometric analysis of sulfur mustard adducts formed with the protein.

IV.5.2 Isolation, purification and enzymatic hydrolysis of keratin from human callus that was exposed to sulfur mustard

First, keratin was isolated from human callus by salt extraction and was subsequently purified by means of gel filtration according to procedures reported in literature (22). The amino acid composition of the isolated keratin was in reasonable agreement with literature data (23; see Table 3).

Table 3. Amino acid composition (mole%) of isolated keratin

Amino acid	isolated keratin	literature data[a]
Gly	23.4	20.9
Ser	13.4	12.4
Glx	9.5	13.3
Asx	8.9	8.5
Leu	7.8	8.4
Thr	6.1	3.7
Lys	5.9	4.5
Arg	5.1	5.5
Ile	4.5	4.0
Ala	4.0	5.5
Phe	3.6	3.2
Val	3.2	3.7
Pro	1.4	1.3
Met	1.2	1.5
His	0.9	1.5
Tyr	0.9	3.6
Cys	not determined	not determined

[a] Reference 23.

Subsequently, a suspension of human callus (0.5 g/ml) in 0.9% NaCl/isopropanol (1/1, v/v) was exposed to various concentrations of [^{14}C]sulfur mustard for 6 h at 37 °C. The extracted keratin fractions contained ca. 15-20% of the added radioactivity, in each case (see Table 4). Upon purification on a G-75 column ca. 25% of the activity bound to keratin was eluted with a Tris buffer (10 mM Tris.HCl, 10 mM dithiothreitol, 0.5% SDS, pH 7.6) as low molecular material, probably thiodiglycol.

Several proteases, i.e., trypsin, α-chymotrypsin and V8 protease, were used in order to identify alkylated sites in keratin after exposure of human callus to sulfur mustard. Keratin isolated from human callus that had been exposed to ^{14}C-sulfur mustard was suspended (3.5 mg/ml) in Cleveland's buffer (125 mM Tris.HCl, 0.5% SDS, 10% glycerol, pH 6.8), a borate buffer (50 mM KCl, 50 mM disodium tetraborate, 1 mM dithiothreitol, pH 9.2, diluted 50 times with water) or a citrate buffer (10 mM sodium citrate, acidified with aqueous HCl to pH 2.6) and incubated with trypsin, immobilized trypsin, α-chymotrypsin or V8-protease at 37 °C for 1 h. The filtrates obtained after passing the incubation mixtures over a UF-2 filter (cut-off 10 kDa) were analyzed by means of HPLC with radiometric detection. No radioactive peptide material could be detected, indicating that amino acids or peptides containing a sulfur mustard adduct had not been released.

Table 4. Binding of [^{14}C]sulfur mustard to keratin upon treatment of human callus suspended in 0.9% NaCl (1 g/ml) with various concentrations of the agent in an equal volume of isopropanol

Concentration [^{14}C]sulfur mustard (μM)	[^{14}C]sulfur mustard bound to keratin (% of total radioactivity added)
0.1	17
1.0	15
10	15
100	20
1000	20
10,000	22

IV.5.3 Isolation and derivatization of thiodiglycol after alkaline hydrolysis of keratin

Release of radioactivity upon purification at pH 7.6 of keratin that was exposed to [^{14}C]sulfur mustard (see previous subsection) suggests that part of the adducts formed with keratin are readily split from the protein. It is known that keratin contains a large number of glutamic and aspartic acid residues (cf. Table 3). Consequently, it can be expected that upon exposure to sulfur mustard these residues are converted into esters of thiodiglycol which are readily hydrolyzed with mild base. To check this hypothesis a purified keratin sample, isolated from human callus exposed to ^{14}C-sulfur mustard, was incubated with aqueous NaOH (0.5 M). After chromatography of the mixture on a G75 column, only 20% of total radioactivity coincided with keratin, whereas 80% of total radioactivity eluted as material with low molecular mass. One of the fractions containing the low molecular material was further worked-up with a combined ChemElut/Sep-Pak C18 extraction. TLC analysis with radiometric detection of the obtained extract (in ethyl acetate) showed that the radioactive component coeluted with thiodiglycol. In a later stage, the reaction mixture was filtered over a UF-2 filter (cut-off 10 kDa) for isolation of thiodiglycol, which is a less laborious procedure. It was established that only small losses (< 10%) of [^{14}C]thiodiglycol occurred during filtration and subsequent evaporation of the filtrate to dryness.

These results open the way for sensitive mass spectrometric detection of sulfur mustard exposure of skin, since a method for derivatization of thiodiglycol and subsequent sensitive analysis of the derivative has been reported in literature (24). In preliminary experiments, thiodiglycol obtained from keratin that was exposed to [^{14}C]sulfur mustard was derivatized with pentafluorobenzoyl chloride according to this procedure. HPLC analysis with radiometric detection revealed one single radioactive compound, which coeluted with a synthetic standard of the bis(pentafluorobenzoyl) ester of thiodiglycol.

IV.5.4 Synthesis of haptens containing a glutamic acid or aspartic acid-sulfur mustard adduct

Since we found that glutamic acid and aspartic acid residues in keratin are efficiently alkylated by sulfur mustard (see previous subsection), we intended to synthesize partial sequences of keratin, containing a sulfur mustard adduct of these amino acids, which can be used as haptens

for raising antibodies. It can be expected, however, that the thiodiglycol esters are not stable during immunization. Therefore, we decided to employ the corresponding amides. In the first instance we tried to synthesize these compounds by solid-phase synthesis of a peptide containing a glutamic or aspartic acid residue protected with an allyl function, which can selectively be removed by reduction on a palladium catalyst. Subsequent coupling with 2-(2-aminoethylthio)ethanol would yield the desired compound. However, we did not succeed in removing the allyl function from the peptide when it was still attached to the solid support, despite several efforts, using palladium catalysts of various manufacturers.

We then decided to synthesize a building block which could be incorporated into the peptide during solid-phase synthesis. As starting material we chose the commercially available Boc-Glu-OtBu and Boc-Asp-OtBu, containing a free side-chain carboxylic acid group. Coupling with 2-(2-aminoethylthio)ethanol under the agency of PyBOP and NMM afforded, after purification on silica gel, the glutamine/asparagine amide derivatives in moderate yield. Deprotection with TFA, followed by introduction of the Fmoc group and purification by means of gel filtration on Sephadex LH-20 gave the desired building blocks for solid-phase synthesis. The following peptides derived from partial sequences of human keratins K5 and K14 were synthesized with these building blocks:

1. G-V-V-S-T-H-Q(Nω-HETE)-Q-V-L-R-T-K-N-K, derived from human keratin K14
2. G-I-Q-Q(Nω-HETE)-V-T-V-N-Q-S-L-L-T-P-L-N-K, derived from human keratin K5
3. G-V-M-N(Nω-HETE)-V-H-D-G-K-V-V-S-T-H-E-K, derived from human keratin K14

Electrospray MS analysis showed the correct mass in each case. The peptides will be used for the raising of antibodies. The three native sequences were also synthesized and will serve as reference compounds in immunochemical experiments with antibodies raised against the three Nω-HETE-glutamine or Nω-HETE-asparagine containing peptides.

V DISCUSSION

Synthesis of radioactively labeled sulfur mustard

Radioactively labeled sulfur mustard has advantageously been used in various series of experiments described in this report. In previous studies we synthesized the ^{35}S-labeled agent for similar purposes. However, the synthesis of [^{35}S]sulfur mustard was often accompanied with difficulties which probably resulted from the presence of impurities in the synthesized hydrogen [^{35}S]sulfide. A more reliable synthetic route could be developed for radioactively labeled sulfur mustard containing a ^{14}C-label instead of a ^{35}S-label. An additional advantage of this new product is the much longer half life of its radioisotope. The crucial step in the new synthetic route is the reaction of [^{14}C]bromoethanol with 2-mercaptoethanol. The major by-product formed in this reaction is the disulfide analogue of thiodiglycol. Since no radioactive precursor is involved in the formation of this by-product, it has no influence on the radiochemical purity of the end product.

Development of two Standard Operating Procedures for determination of sulfur mustard adducts

Within the framework of a previous grant (7) two methods for diagnosis and dosimetry of exposure to sulfur mustard were sufficiently worked out to justify the development of a standard operating procedure (SOP) to be applied in a well-equipped field hospital, i.e., an immunoslotblot assay and/or ELISA of sulfur mustard adducts to DNA in human blood and skin, and a GC-NCI/MS determination of sulfur mustard adducts to the N-terminal valine in hemoglobin of human blood by using the modified Edman procedure. Development of these SOPs is one of the two major topics of the present grant and will be performed in three phases: (i) simplification and optimization of the two methods, (ii) validation in animal experiments of the methods performed according to the final procedures, and (iii) description of the SOPs and performance of the procedures in a U.S. Army laboratory in order to demonstrate their practical applicability.

During the period covered by this first annual report, studies relating to the first item, i.e., simplification and optimization of the methods, were performed. Two approaches have been taken for optimization of the two methods. On the one hand, experiments were performed aiming at a procedure that is most suitable for performance in a field laboratory, i.e., a procedure as simple as possible which can be carried out in a relatively short period of time. On the other hand, the procedure has been modified in order to achieve a lower detection limit for optimum analysis of samples that can be sent to a well-equipped research laboratory.

Simplification of the immunochemical assay

The modifications applied so far were meant to simplify and to speed up the procedures for isolation and processing of DNA and for an immunoslotblot assay while maintaining maximum sensitivity. In a later stage, modifications will be introduced to speed up the first steps followed by an ELISA assay, aiming at a procedure as simple as possible while accepting some decrease in sensitivity and accuracy.

The present experiments showed that several steps could be simplified and minimized. The steps involved are: collection of a blood sample and/or skin biopsy, the isolation of DNA, measurement of the concentration and denaturation of DNA, followed by the immunoslotblot procedure, involving blotting and crosslinking of the DNA on a nitrocellulose filter, a blocking

step, treatment with 1st and 2nd antibody, addition of substrate and, finally, measurement of the chemiluminescence as a measure for the amount of sulfur mustard adducts to the DNA. The amount of blood required could be reduced to only 300 µl. In addition, sufficient amounts of DNA could be isolated from a skin biopsy of 10-20 mm^2.

Although the DNA isolation procedure is still a time-consuming step, substantial reduction in time and labour could be achieved. When the analysis can be carried out on fresh blood, it is estimated that the whole DNA isolation procedure was reduced from one working day to about 4 h in which some variation in time may occur in the final dissolution step of the DNA precipitate. In this period about 20 samples can be handled simultaneously. In practice, the immunochemical assay may not be carried out on the same day as the blood samples or skin biopsies have been collected. In that case, the samples can be stored in a freezer. However, one has to keep in mind that the procedure for isolation of DNA from frozen blood samples takes more time (sometimes a night) due to the slow dissolution of the DNA after the final precipitation step.

Finally, the use of a luminometer for the direct measurement of the chemiluminescence, instead of the combination of a photographic film and a densitometer for measurement of the blackening, appeared to yield a significant reduction in time and labour.

In the present set up of the immunoslotblot procedure, 39 samples can be assayed in duplicate on one nitrocellulose filter, in addition to the standard DNA samples.

The immunoslotblot procedure in its current state, including the DNA isolation procedure, takes about 1.5 working days, which is mainly due to the overnight adsorption step of the 1st antibody. In an alternative assay, incubation with the 1st antibody was carried out for 2 h at 37 °C. This resulted in a lower sensitivity. The extent of impairment is still under study.

Sensitivity of the immunochemical assay

In addition to the modifications which resulted in simplification and shortening of the assay, several improvements could be achieved with respect to the sensitivity. The accurate measurement of the concentration of DNA appeared to be essential due to the strong dependence of the chemiluminescence signal upon the amount of DNA blotted on the filter. The UV crosslinking on the nitrocellulose filter resulted in an about 10-fold enhancement of the chemiluminescence signal which had direct consequences for the sensitivity of the assay. The use of a luminometer for measurement of chemiluminescence instead of exposure to a photographic film circumvents the non-linear blackening characteristics of the films. In this way a linear relation was obtained for the chemiluminescence as a function of the sulfur mustard adduct concentration.

As a result of these modifications, chemiluminescence observed for DNA treated with 2.5 nM sulfur mustard was enhanced relative to that for untreated DNA, whereas the lower detection limit in previous experiments was at about 10 nM sulfur mustard. The lower detection limit in the modified assay showed some variation which may partly be due to day-to-day variations in the state of our chemiluminescence blotting detection system of Boehringer. Nevertheless, it could be derived that the lower detection limit was in a range of 8-40 amol N7-HETE-Gua/blot with 1 µg DNA. This corresponds to an adduct level of 3-13 N7-HETE-Gua/10^9 nucleotides.

At the start of this study the lower detection limit of the immunoslotblot assay for exposure of human blood was 70 nM sulfur mustard. This corresponds to an adduct level of 300 N7-HETE-Gua/10^9 nucleotides. Due to the improvements mentioned above, it can be derived that the lower

detection limit for *in vitro* exposure of human blood should be 0.7-3 nM sulfur mustard. However, the adduct levels detected at the lower sulfur mustard concentrations were much lower than expected, for unknown reasons. It is currently under study whether this is of artificial origin or not.

Simplification and optimization of a GC-NCI/MS determination of the sulfur mustard adduct to the N-terminal valine in hemoglobin

The first step in the procedure is the isolation of globin from blood, which takes approximately 2-3 hours. Unfortunately, a straightforward approach of shortening the procedure by omitting this isolation step and treating the hemolysate with the modified Edman reagent did not lead to a detectable product. However, the duration of the second step, i.e., treatment with the modified Edman reagent, could be considerably diminished without loosing sensitivity by performing the reaction for 2 h at 60 °C instead of overnight at room temperature, followed by 2 h at 45 °C. In addition, the work-up of the reaction mixture was somewhat shortened. As a result, the whole procedure, i.e., isolation of globin, reaction with the modified Edman reagent, work-up, derivatization, and GC-NCI/MS analysis, can now be performed within one working day.

In vitro exposure of human blood to ≥ 0.1 µM sulfur mustard could be determined by using the modified Edman procedure as developed in our previous studies (7). This method was sufficiently sensitive for retrospective detection of exposure to sulfur mustard of victims from the Iran-Iraq conflict (12). Nevertheless, some steps in the procedure can presumably be improved leading to an enhancement of the sensitivity, e.g., the processing of a larger sample of globin isolated from blood that was exposed to sulfur mustard, and GC-NCI/MS analysis of a larger fraction of the final sample obtained by the procedure. In a first series of experiments solid phase extraction procedures were used to purify the crude thiohydantoin obtained after treatment with the modified Edman reagent. It may be assumed that the substantial purification achieved will allow us to process larger amounts of globin and consequently to detect lower exposure levels. In a second series of experiments, a TCT injection technique was used in the GC-NCI/MS analysis of the final sample. Much larger injection volumes can be applied by using this technique instead of a normal on-column injection (50-100 µl vs 1-3 µl), so far leading to a 3-fold decrease of the detection limit for in vitro exposure of human blood. This unexpectedly small increase in sensitivity may be due to incomplete desorption from the Tenax material on which the sample is applied or to decomposition during desorption. This will be further investigated. Nevertheless, it is assumed that the sensitivity of the modified Edman procedure can be improved by at least one order of magnitude, since both approaches lead to positive results. A further evaluation will be performed in the second year of this grant.

Further exploratory research on immunochemical assays of protein adducts

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Whereas in human skin most of the N7-HETE-Gua has disappeared two days after *in vivo* exposure (7), it is expected that adducts to proteins have life-spans varying from several weeks up to a few months (25). Consequently, the retrospectivity of the diagnosis on the basis of protein adducts is superior to that on the basis of DNA adducts. Moreover, detection is supposedly also more sensitive in case of a long-term exposure to sulfur mustard at low concentrations. Therefore, antibodies were raised against S-HETE-cysteine in partial sequences of human hemoglobin in our previous studies (7). However, the lower detection limit obtained for in vitro exposure of human blood with these antibodies was only 50 µM of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts is the second major topic of the present grant, in addition to the development of standard operating procedures.

Information on immunochemical assays for the detection of protein adducts is only scarcely available in literature. In general, the antibodies have been generated by using adducted keyhole limpet hemocyanin or albumin as an immunogen. These immunogens were obtained by a direct coupling of the reactive compound to the protein, e.g., for 7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (26), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (27), diclofenac (28), and 4-hydroxy-2-nonenal (29), or by coupling of the adducted amino acid corresponding to the adducted site in the protein, e.g., 3-(N-acetyl-cystein-S-yl)-acetaminophen (30). A similar approach was followed in our previous study (7) by using sulfur mustard treated keyhole limpet hemocyanin and hemoglobin as immunogen. However, these experiments did not result in antibodies recognizing adducts in sulfur mustard-treated hemoglobin.

In a more systematic approach haptens having sequential similarity to parts of the adducted protein surface were used for raising antibodies. This approach was only followed in a few studies. Lin et al. (31) generated antibodies against two partial sequences of hemoglobin both adducted with acetaldehyde at lysine residues. Wraith et al. (32) used the N-terminal heptapeptide of α-globin hydroxyethylated at the terminal amino group as a hapten for raising antibodies against hemoglobin exposed to ethylene oxide. An immunoassay based on the antibody obtained and GC-MS analysis following modified Edman degradation showed comparable results and sensitivities.

A similar approach is followed in the present study. Partial sequences of a protein are synthesized as haptens, which are based on mass spectrometric identification of adducted amino acids in the protein. The results obtained in the mass spectrometric analyses also provide guidance as to which amino acids should be used for quantitative GC-MS or LC-MS-MS analysis, in order to verify immunochemical assays. Investigations are performed on three proteins, i.e., hemoglobin, albumin, and keratin. The accessibility of the adducts for immunochemical analysis is supposed to increase in this order, i.e., hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma, whereas keratins in the skin are directly accessible for sulfur mustard and for reagents.

Detection of hemoglobin adducts

Characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin

Antibodies (clone 3H6) raised against an alkylated peptide, i.e. N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin, were further characterized. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA. However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have now attempted to apply these antibodies in an immunoslotblot assay to alkylated hemoglobin, but without lowering the detection limit so far. Several other clones were obtained from the above-mentioned immunization which produced antibodies that recognize alkylated hemoglobin. These are currently being characterized.

Antibodies against peptide haptens containing a histidine-sulfur mustard adduct

N1/N3-HETE-Histidine is the most abundant amino acid adduct formed in hemoglobin after exposure of human blood to sulfur mustard (7). In addition, three out of the five sites of alkylation within the tertiary structure of hemoglobin identified from electrospray tandem MS analyses in tryptic digests of globin isolated from human blood that was exposed to sulfur mustard are histidine residues, i.e., α-his_{20}, β-his_{77} and β-his_{97}. Therefore, partial sequences of

hemoglobin containing these adducted amino acids were synthesized as haptens for raising antibodies.

In our previous studies (7), synthesis of N1/N3-HETE-histidine-containing peptides on a solid support was not successful when using the adducted amino acid unprotected at the 2-hydroxyethylthioethyl group. We have now synthesized a properly protected building block starting from Nα-Boc-N1/N3-*tert*-butyloxyethylthioethyl-L-histidine methyl ester, which was previously obtained by using the semi-mustard derivative 2-(2-*tert*-butyloxyethylthio)ethyl chloride for introduction of a protected 2-hydroxyethylthioethyl group (7). The three partial sequences of hemoglobin could conveniently be synthesized on a solid support by using this building block.

We immunized mice with these three different peptide haptens. From all three haptens clones were obtained producing antibodies with specificity for hemoglobin treated with 50 µM sulfur mustard. In the same experiment, the control clone, 3H6, appeared to be negative which suggests that the test system, particularly the coating of the microtiter plates, was not optimal. Nevertheless, several other clones were still positive, suggesting that these clones produced antibodies which were more specific than those of 3H6. Antibodies of these clones show specificity not only for alkylated hemoglobin but also for alkylated keratin. In the case of clone 190-2H12, the specificity for alkylated keratin seemed to be even higher than for alkylated hemoglobin. This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Phosphate mono-esters exhibit high reactivity towards mustard agents (33). Because the termini of keratins contain (inexact) repeats of glycine and (phospho)serine residues we also examined, in a parallel study to this Grant Agreement, the formation of alkylated phosphoserine residues in keratin from human epidermis. Two peptides both containing a phosphoserine-sulfur mustard adduct were synthesized (34) and served as haptens for raising antibodies. Several clones have been obtained producing antibodies not only specific for alkylated (phosphoserine-containing) keratin but also for alkylated hemoglobin. Some clones produced antibodies which were even more specific for alkylated hemoglobin than for alkylated keratin. This suggests once more that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Detection of albumin adducts

An electrophilic compound has to cross the cell membrane of the erythrocyte in order to react with hemoglobin. Therefore, adduct formation with plasma proteins might be more efficient (25). The most abundant plasma protein is albumin, which has a relatively slow turn-over in human beings (half-life 20-25 days). Covalent binding of ultimate carcinogens derived from various compounds has been documented, e.g., benzene (17), benz(a)pyrene (35), 2-amino-3-methyl-imidazole[4,5-f]quinoline (36), and aflatoxin B1 (37).

In a first series of experiments, the extent to which albumin is alkylated by sulfur mustard was investigated by using ^{14}C-labeled agent. It was found that a proportional amount of sulfur mustard (ca. 20%) was bound to albumin isolated from human blood treated with various concentrations (1.3 µM - 1.3 mM) of the agent, indicating a linear relationship between exposure concentration and adduct level. Similar results were previously obtained for binding to hemoglobin (7). Although the latter protein is enclosed in erythrocytes, it binds an even somewhat greater fraction of the agent (ca. 25%). These in vitro experiments show that binding

to albumin and hemoglobin accounts for almost 50% of the total elimination of sulfur mustard, when introduced into human blood.

In order to identify adducted amino acids, tryptic digests of albumin isolated from blood that had been exposed to [^{14}C]sulfur mustard were analyzed by means of HPLC. Fortunately, these analyses showed one major radioactive fragment which in addition was fully separated from other peptide fragments. Therefore, we have not extensively analyzed sulfur mustard alkylation sites in albumin, but have focused our attention on the major fragment. This fragment was fully identified by LC-MS-MS analysis of the tryptic digest as the T5 peptide of albumin alkylated at cysteine-34. In addition, this alkylated heneicosapeptide synthesized on a solid support coeluted with the major fragment upon HPLC analysis. Cysteine-34 has previously been identified as a nucleophilic site capable of reacting with electrophiles (17). It is the only reactive sulfhydryl group in the protein.

Based on these results, it seems worthwhile to use the synthetic alkylated peptide as a hapten for raising antibodies against sulfur mustard treated albumin as well as to investigate whether quantitative mass spectrometric analysis of this peptide in a tryptic digest of albumin is suitable for retrospective detection of exposure to sulfur mustard. These investigations will be performed in the second and third year of the grant period.

During the current period, a first series of experiments on quantitative determination of the alkylated T5 peptide has been performed. By analyzing this peptide in a tryptic digest, the tedious complete hydrolysis of the protein and the subsequent work-up of a specific adducted amino acid in the hydrolysate can be circumvented. The detection limit for in vitro exposure of human blood to sulfur mustard was found to be 1 μM by LC-MS-MS analysis under MRM conditions of the tryptic digest, which was not further worked up. Enhancement of the sensitivity was hampered by small signals observed in the blank samples at the same retention time as the analyte. Some of the analyses were performed in U.K. by using a Q-TOF-MS. Since this technique allows to acquire a full scan MS-MS spectrum of the peptide analyte at the same absolute sensitivity as provided by an electrospray MS-MS analysis under MRM conditions, its sensitivity for analysis in biological samples may be enhanced due to a higher potential specificity. A Q-TOF-mass spectrometer will become available in TNO Prins Maurits Laboratory in the near future. A quantitative mass spectrometric determination of the alkylated T5 fragment in tryptic digests of exposed albumin will be further developed in the second year of the grant period, including investigations on the possibilities offered by the Q-TOF-MS.

Detection of keratin adducts

The skin is a major target for vesicants such as sulfur mustard. To the best of our knowledge, adducts of alkylating agents with proteins present in the skin have not been studied so far. However, proteins in the skin, particularly those in the stratum corneum, are readily accessible for agents. The most abundant protein in stratum corneum and epidermis is keratin. Therefore, methods for retrospective detection of skin exposure to sulfur mustard are being developed in the present study by analyzing adducts formed with this protein.

The cytoskeleton of most mammalian cells includes a network of 8 to 10 nm filaments called intermediate filaments (IFs) (38,39). Keratins (MW 40-70 kDa) form the backbone of the IFs in epithelial tissues. In basal epidermal cells almost 30% of all synthesized proteins are keratins. Their structures are closely related and can be represented by a central α-helix rich domain (length 300-350 residues) flanked on either side by non-helical domains of variable size and chemical character. The helical segments contain heptad repeats of hydrophobic residues. In

addition, a conserved periodic distribution of acidic (aspartic acid, glutamic acid) and basic (arginine, histidine and lysine) amino acids is found in IFs. The termini contain *inter alia* inexact repeats of glycine and (phospho)serine residues. Amino acid sequences of a number of human keratins have been documented (see for instance ref. 38). Most data indicate that the end domains are predominantly located on the surface of the IFs.

In the first series of experiments, the binding to keratin was quantitated and attempts were made to identify adducted amino acids formed in keratin. The experiments were performed with human callus as a model for human skin, which was suspended in a solution of [^{14}C]sulfur mustard. The amount of the agent bound to keratin (15-20%) was proportional to the concentration used of sulfur mustard (0.1 μM - 10 mM), as was found for hemoglobin and albumin after exposure of human blood to the agent. Identification of the adducted amino acids was hampered by the absence of radioactively labeled amino acids or peptides in HPLC analyses of the lower molecular fraction obtained by filtration (cut-off 10 kDa) of incubation mixtures of exposed keratin with various proteases, i.e., trypsin, α-chymotrypsin and V8 protease.

It was found that ca. 25% of the radioactivity was split off upon gel filtration of exposed keratin at pH 7.6, whereas even 80% of total radioactivity eluted as material with low molecular mass by gel filtration of exposed keratin that was treated with aqueous NaOH (0.5 M), which was identified as thiodiglycol. These results suggest strongly that most of the adducts formed with keratin are esters of thiodiglycol with glutamic and aspartic acid residues, which are readily hydrolyzed with (mild) base. Therefore, we focused our attention on analysis of sulfur mustard adducts with these two amino acid residues for the development of both an immunochemical and a mass spectrometric method for retrospective detection of exposure of skin to sulfur mustard.

For development of an immunochemical assay, two partial sequences of keratin K14 and one partial sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies. The peptides contain adducted glutamine or asparagine instead of the corresponding thiodiglycol esters with glutamic acid and aspartic acid. The resulting amides are expected to be stable during immunization in contrast to the thiodiglycol esters. Initially, it was attempted to form the adducted amino acid residues on a solid support after selective removal of a protecting allyl function from the glutamine or asparagine residue when the peptide was still attached to the solid support. However, we did not succeed in splitting off the protecting group at these conditions. We then synthesized properly protected building blocks for the two adducted amino acids, analogously to the approach followed for the synthesis of N1/N3-HETE-histidine containing peptides on a solid support (vide supra). Also in this case, the desired peptides could conveniently be synthesized by using these synthons.

Mass spectrometric analysis of thiodiglycol that is released by mild base from keratin exposed to sulfur mustard will be an attractive method for retrospective detection of skin exposure. The analyte is simply obtained without degradation of the protein, which avoids generation of other low molecular material. In addition, the ready release of thiodiglycol from sulfur mustard exposed keratin opens the way for direct detection of adducts in the skin without taking biopsies, by spraying the skin with appropriate reagents. In preliminary experiments to develop a mass spectrometric method, it was demonstrated that thiodiglycol could efficiently be isolated by filtration and subsequent concentration after alkaline treatment of keratin that was exposed to sulfur mustard. Furthermore, the isolated thiodigycol could be derivatized with pentafluorobenzoyl chloride according to a procedure reported in literature (24). This

procedure and GC/MS analysis of the derivative will be further worked out during the second year of the grant period.

VI CONCLUSIONS

1. A convenient route for synthesis of [^{14}C]sulfur mustard was developed, which leads to more reproducible results than synthesis of the ^{35}S-labeled agent.

2. The first steps of an immunoslotbot assay of sulfur mustard adducts to DNA in human blood and skin, i.e., DNA isolation and processing, could substantially be reduced in time and labour, down to ca. 4 h.

3. A significant reduction in time and labour for performing an immunoslotblot assay was achieved by using a luminometer for measurement of the chemiluminescence instead of the combination of a photographic film and a densitometer for measurement of the blackening. Moreover, a linear relationship was obtained between observed chemiluminescence and sulfur mustard adduct concentration.

4. The lower detection limit of the modified immunoslotblot assay was reached at 8-40 amol N7-HETE-Gua/blot with 1 µg DNA, corresponding to an adduct level of 3-13 N7-HETE-Gua/10^9 nucleotides. The lower detection limit for treatment of double stranded calf thymus DNA with sulfur mustard was 2.5 nM.

5. The modified Edman procedure for determination of sulfur mustard adducts to the N-terminal valine in hemoglobin including GC-NCI/MS analysis can be shortened to one working day without loosing sensitivity, by performing the Edman degradation reaction for 2 h at 60 °C instead of overnight at room temperature followed by 2 h at 45 °C.

6. A substantial purification of the crude thiohydantoin was achieved by introducing a solid phase extraction step into the modified Edman procedure, which may allow us to process larger amounts of globin and consequently to detect lower exposure levels.

7. Application of a TCT injection technique in the GC-NCI/MS analysis of the final sample obtained after the modified Edman procedure led to a 3-fold decrease of the detection limit for in vitro exposure of human blood.

8. A convenient route has been worked out for synthesis of a properly protected building block of N1/N3-HETE-histidine that is suitable for solid phase synthesis of peptides.

9. Partial sequences of hemoglobin containing an adduct with a histidine that was previously identified as an alkylation site for sulfur mustard, i.e., α-his$_{20}$, β-his$_{77}$ or β-his$_{97}$, were synthesized as haptens for raising antibodies against sulfur mustard treated hemoglobin.

10. Several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with 50 µM sulfur mustard but also for alkylated keratin.

11. A proportional amount of [^{14}C]sulfur mustard (ca. 20%) was bound to albumin isolated from human blood treated with various concentrations of the agent.

12. HPLC analysis of tryptic digests of albumin isolated from blood that had been exposed to [^{14}C]sulfur mustard showed one major fragment which was fully separated from other peptide fragments and was fully identified by LC-MS-MS analysis as the T5 fragment of albumin alkylated at cysteine-34.

13. The alkylated T5 peptide of albumin was synthesized on a solid support as a hapten for raising antibodies against sulfur mustard treated albumin.

14. So far, LC-MS-MS analysis under MRM conditions performed directly on the T5 fragment in a tryptic digest of albumin that was isolated from sulfur mustard treated human blood allowed to detect exposure to 1 μM of the agent.

15. The amount of [^{14}C]sulfur mustard bound to keratin in human callus (15-20%) was proportional to the concentration of the agent in the solution in which human callus had been suspended.

16. Most of the adducts formed with keratin in human callus that was exposed to [^{14}C]sulfur mustard are esters of thiodiglycol with glutamic and aspartic acid residues, since treatment of exposed keratin with aqueous NaOH (0.5 M) released 80% of the total radioactivity, which was identified as thiodiglycol.

17. Thiodiglycol that was released by treatment with base from keratin exposed to sulfur mustard was readily isolated and derivatized with pentafluorobenzoyl chloride, allowing GC-MS analysis.

18. Two partial sequences of keratin K14 and one partial sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, by using a properly protected building block of glutamine or asparagine adducted with a 2-hydroxyethyl-thioethyl group at the amide function.

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